Detection of *Northern Cereal Mosaic Virus* mRNAs encoding ORFs 3 to 6 in Plant and Insect Tissues.

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Summary

The genome of *Northern Cereal Mosaic Virus* (NCMV), a plant rhabdovirus, has a negative sense RNA genome potentially encodes nine ORFs in the order; 3' N-P-3-4-5 -6-M-G-L 5', where the genes 3 to 6 are unique to NCMV. We examined the expression of NCMV genes 3, 4, 5 and 6 in plant and insect host cells by 3'RACE analysis. PCR product with the size corresponding to each of these four genes was amplified. However, amplification of the gene 3 specific transcripts consistently yielded two fragments of distinct lengths. Nucleotide sequence and northern blot analyses showed the large transcript (0.9kb) represents premature termination product consisting of *gene3* and *gene4* regions, whereas the small transcript is the *gene3* mRNA.

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Key words : NCMV, Rhabdovirus

Northern Cereal Mosaic Virus (NCMV), a member of the genus *Cytorhabdovirus* of the family *Rhabdoviridae*, is an enveloped virus with a non-segmented negative sense genome RNA (Dietzen *et al*., 2011). NCMV infects and causes serious symptoms (chlorotic spot, stripe, rosette stunt) in cereals (wheat, barley, oats) in northern Japan, Korea and China (Toriyama 1972, 1986; Kojima *et al*. 1985), and is transmitted in a persistent and propagative manner by planthopper, *Laodelphax striatellus*. The complete genome sequence of 13,222 nucleotides (nts) was determined for Tokyo isolate of NCMV (Tanno *et al*. 2000). The genome has the 5' trailer sequence 273nts and the 3' leader sequence 90nts. A characteristic intergenic sequence separates each gene, the gene order is; 3' N-P-3-4-5-6-M-P-G-L 5', including the five structural protein genes (N, P, M, G, L) conserved among other rhabdoviruses (Fig.1. A)(Kurath *et al*. 1985, Tordo *et al*. 1986, Heaton *et al*. 1989, Witzel *et al*. 1994, Pringle and Easton 1997, Dietzgen *et al*. 2011). Individual virus genes are transcribed by virus encoded RNA dependent RNA polymerase (L protein) from the genome RNA template. Proteins corresponding to NCMV genes3-6 were not identified in the virion proteins (Shirako *et al*.1985), and their functions have not known.

Although the biological, epidemiological and structural properties of NCMV have been extensively studied (Toriyama, 1972; Shirako and Ehara, 1985; Toriyama, 1986), little is known about its genome expression and replication strategy. In this paper we report on identification of NCMV mRNAs encoding the ORFs 3, 4, 5 and 6 in plant and insect host cells.

Materials and Methods

The virus and RNA extraction

The Tokyo isolate of NCMV was maintained in barley plants (*Hordeum Vulgare* L.,cv. Hamayutaka) by planthopper-mediated transmission (Toriyama 1972). When mosaic symptoms were developed (about 2 weeks after inoculation), insects and infected barley leaves were collected and stored for assay. Individual adult insects were ground with mortar and pestle, homogenized in 0.25 ml of ISOGEN (Nippon gene Co. LTD). The extraction of total RNA from viruliferous planthoppers was according to the manufacturer's instructions. Total RNA from NCMV infected leaves (100mg) were prepared using RNeasy plant total RNA kits (QIAGEN).

The 3' RACE procedure

Total RNA extracts from plant or insect hosts were used as the templates for cDNA symthesis with reverse transcriptase (Gibco BRL) and oligo-dT M4 primer (TAKARA) (Table 1). The cDNA mixture was used as a template for the PCR amplification. Based on the nucleotide sequence of the NCMV genome, the specific forward primer including the ATG initiation codon of each ORF of genes 3 to 6 (Table 1., shown in bold) was designed. PCR reactions were performed with Ex Taq polymerase (TAKARA) using the gene-specific primer and M13-M4 primer. The PCR reaction

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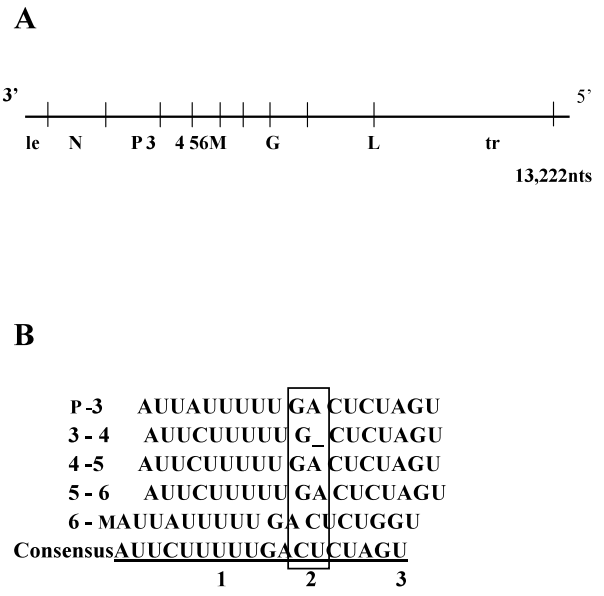

Fig 1.NCMV genome organization and intergenic region sequences

A.Organization of the NCMV genome. le: 3'leader sequence, N: nucleocapsid protein gene, P: phosphoprotein gene, 3 - 4 - 5 - 6: gene3, 4, 5, 6, respectively, M: matrix protein gene, G: glycoprotein gene, L: large (polymerase) protein gene, tr: 5'trailer sequence.

B.Comparison of the intergenic region sequences separating individual genes on the NCMV genome (Tanno et al. 2000). The underlined motifs refer to (1) the putative polyA signal, (2) the putative gene junction dinucleotides GA, and (3) the putative transcription start site..

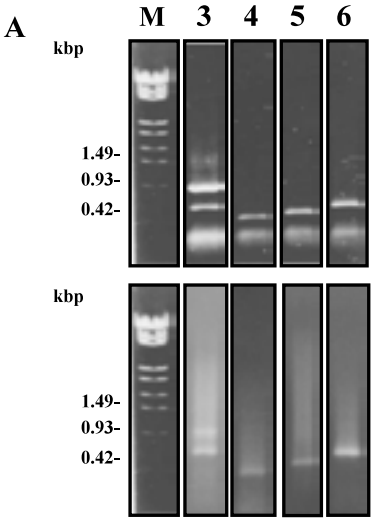

Fig 2. 3' RACE of NCMV mRNA for gene 3 to gene 6

M: DNA size maker (λ -DNA/ Sty I), lane number is consistent with gene name. Amplification products from NCMV from infected barley (A), NCMV from viruliferous planthopper (B).

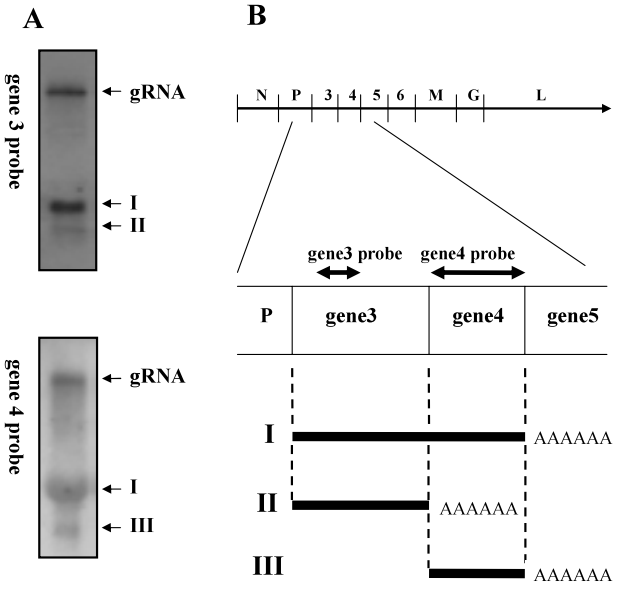

Fig 3. Northern blot hybridization of gene3 and gene4 (A), and proposed transcription map (B)

A: Detection of mRNA for gene 3 and gene 4. Total RNA from viruliferous insects was fractionated on 1% agarose-formaldehyde gel, transferred to nylon membrane, and hybridized with DNA probe specific to gene 3 and gene 4 regions. gRNA indicates viral genome RNA. Symbols I, II, III denote major transcripts; transcript containing *gene 3* and *gene 4* (I), mRNA of *gene 3* (II) and *gene 4* (III), respectively.

B: Schematic diagram showing genome organization of NCMV, location of gene specific probes, and three major transcripts of gene3 and gene4 regions. PolyA is represented by "AAAAAA".

was conducted under the temperature program: 95℃/ 30 sec, 50-56℃/30 sec, 72℃/40 sec (30 cycles). The PCR products were electrophoresed on TBE- agarose gel (1%).

Some PCR products were labeled by a Dye-terminator cycle sequencing kit and sequenced by an automated 373A DNA sequencer (Applied Biosystems).

Northern blotting

Total RNA preparations from viruliferous planthoppers were separated by electrophoresis on 1% formaldehyde-MOPS agarose gel and transferred to a Hybond-N+ membrane (Amersham-Pharmacia). The probes were designed as fellows : (1) cDNA clone pR19/Pst I fragment, position 2642-2878nts, was used as the gene 3 specific probe, (2) Amplified fragment derived from 3'RACE of the gene4 was used as the gene 4 specific probe. These DNA fragments were labeled by the Gene Images labeling and detection system (Amersham-Pharmacia).

Results and Discussion

The PCR products with sizes expected for the genes 3 to

Table 1. Oligonucleotide DNA primers used in present study

primer	position (nts)	5' sequence 3'
ORF3	2500-2527	ATGTCTTCGAAATTCGGAA
ORF4	3035-3053	ATGCCGTGCTGTGTAATA A
ORF5	3401-3479	ATGGATGAGTTAAGGTTAA
ORF6	3815-3873	ATGGCCGCCGTTCTCGTTG
Oligo-dT-M4		GTTTCCCAGTCACGACT(18-20)
M13-M4		GTTTCCCAGTCACGAC

6 were amplified; 530bp in the gene 3, 370bp in the gene 4, 400bp in the gene 5, 510bp in the gene 6, respectively (Fig.2A, B). However, using gene 3-specific primer pair, an additional large DNA fragment of about 900bp was amplified from both plant and insect RNA samples (Fig.A, B, lane3). The gene 3-derived two cDNA bands were separated by gel extraction system, and were cloned into plasmid vector and sequenced. The 525 nucleotide sequence of the small cDNA fragment was mapped to the genome position 2500-3024nts, which spans from the initiation codon to the putative poly A signal of gene 3 (Table 1, B), whereas the 890 nucleotide sequence of the large cDNA was mapped to the position 2500-3389nts, that contained the entire genes 3 and 4, and a short stretch of the putative gene 4 poly A signal (data not shown) .

In northern hybridization, the gene 3 specific probe captured three RNA species, 13kb viral genome RNA (gRNA), 0.9kb large transcript (I) and 0.5kb *gene3* mRNA (II) (Fig.3A). The gene 4 specific probe also hybridized with three RNA bands, gRNA, 0.9kb transcript (I) and 0.3kb *gene4* mRNA (III). These results, together with the 3' RACE and sequence analyses confirmed that the 0.9kb RNA transcripts results from premature termination at gene 3-gene 4 junction (Fig.3. B). The intergenic sequence, 3'-AUUCUUUUUGACUCUAGU-5' was well conserved in NCMV genome. However, the junction between gene 3 and 4 had a nucleotide deletion (Fig.1. B). It is possible that the single A nucleotide deletion in the intergenic sequence between gene 3 and gene 4 is responsible for the leaky termination of gene 3 transcripts. Several papers have been reported that sequence and nucleotide composition of intergenic region alter the efficiency of the start/termination of transcription (Stillman and Whitt 1998, Finke *et al.* 2000). In the case of NCMV, the premature termination transcripts accumulated high level than genuine *gene3* and *gene4* mRNAs (Fig.3.A). Whether the 0.9kb large transcripts have any regulatory role in expression of gene 3 or gene 4 products remains an open question.

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北地ムギモザイクウイルスのORF3から6をコードするmRNAの植物および昆虫組織からの検出

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要　約

北地ムギモザイクウイルス（NCMV）は植物ラブドウイルスの一種で、マイナス鎖RNAゲノム上に、3' N-P-3-4-5-6-M-G-L 5'の順に９個の翻訳読み枠（ORF）が存在するが、このうち３から６のORFはNCMVに固有の遺伝子で、他のラブドウイルスには見られない。NCMVに感染した植物および昆虫組織におけるこれら４種の遺伝子の発現を3'RACE法により調査した結果、植物と昆虫のいずれにおいても推定される４種類のmRNAに対応したサイズのcDNA断片が増幅された。このうちORF3においては、予測されるサイズ0.5kbpの断片の他に、推定サイズ約0.9kbpのcDNAが増幅された。ノーザン解析と塩基配列解析により、サイズの小さな転写物がORF3に対応したmRNAであり、0.9kbの転写物はORF3とORF4領域を含み、両遺伝子間に存在する転写終結シグナルの読み過ごしにより生成したものであることが明らかになった。

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