NUCLEOTIDE SEQUENCES OF THE CYLINDRICAL INCLUSION PROTEIN GENES OF TWO JAPANESE ZUCCHINI YELLOW MOSAIC VIRUS ISOLATES

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Summary. – The nucleotide sequences of the cylindrical inclusion protein (CIP) genes of two Japanese zucchini yellow mosaic virus (ZYMV) isolates (ZYMV-169 and ZYMV-M) were determined. The CIP genes of both isolates comprised 1902 nucleotides and encoded 634 amino acids containing consensus nucleotide binding motif. The sequence similarities between the two isolates at the nucleotide and amino acid levels were 91% and 98%, respectively. When the CIP gene sequences of the Japanese ZYMV isolates were compared with those of previously reported ZYMV isolates, the nucleotide and amino acid sequence similarities ranged between 81% and 97%, and between 95% and 97%, respectively. Phylogenetic analysis of the deduced amino acid sequences of the CIP genes indicated that the Japanese ZYMV isolates were closely related to those of other ZYMV isolates.

Key words: zucchini yellow mosaic virus; Japanese isolates; cylindrical inclusion protein gene; phylogenetic analysis

ZYMV (*Potyvirus* genus, *Potyviridae* family) causes serious losses of cucurbitaceous crops worldwide (Lisa and Lecoq, 1984). Potyviral virion particles are composed of a single-stranded, positive-sense RNA of nearly 10 kb encapsidated by approximately 2000 copies of coat protein (CP) monomers in a helical fashion (Lindbo and Dougherty, 1994). The potyviral genome is covalently bound at the 5'-end to a virus-coded protein designated VPg (Hari, 1981) and has a poly(A) sequence at the 3'-end (Hari *et al.*, 1979). The genome contains a single open reading frame (ORF) that is translated into a large polyprotein and processed co- and post-translationally into eight or more proteins by three virus-coded proteinases (Dougherty and Semler, 1993). Out of these proteins, VPg and CP are the only gene products detected in virus particles. Other gene products, P1-Pro, HC-Pro, P3, CIP, NIa, and NIb but not 6K1 and 6K2 have been detected in infected plants and characterized (Dougherty and Carrington, 1988; Rodríguez-Cerezo and Shaw, 1991).

All potyviruses induce the formation of characteristic „pinwheel„ cylindrical inclusions in the cytoplasm of infected cells (Edwardson, 1974), and this property has been considered one of the most important phenotypic criterion for assigning viruses to the *Potyviridae* family (Shukla *et al.*, 1989). Moreover, the relatively large size of CIP allows for easy detection and is potentially more useful than CP for diagnosis and classification of potyviruses (Yeh and Gonsalves, 1984). The CIP of potyviruses has been shown to be an RNA helicase (Laín *et al.*, 1989), and has a nucleic acid-stimulated ATPase activity (Laín *et al.*, 1991).

Recently, the nucleotide and deduced amino acid sequences of the CP genes of two Japanese ZYMV isolates have been compared with those of previously reported ZYMV isolates (Kundu *et al.*, 1997). In the present study, we have determined the nucleotide and deduced amino acid sequences of the CIP genes of the two Japanese ZYMV iso-

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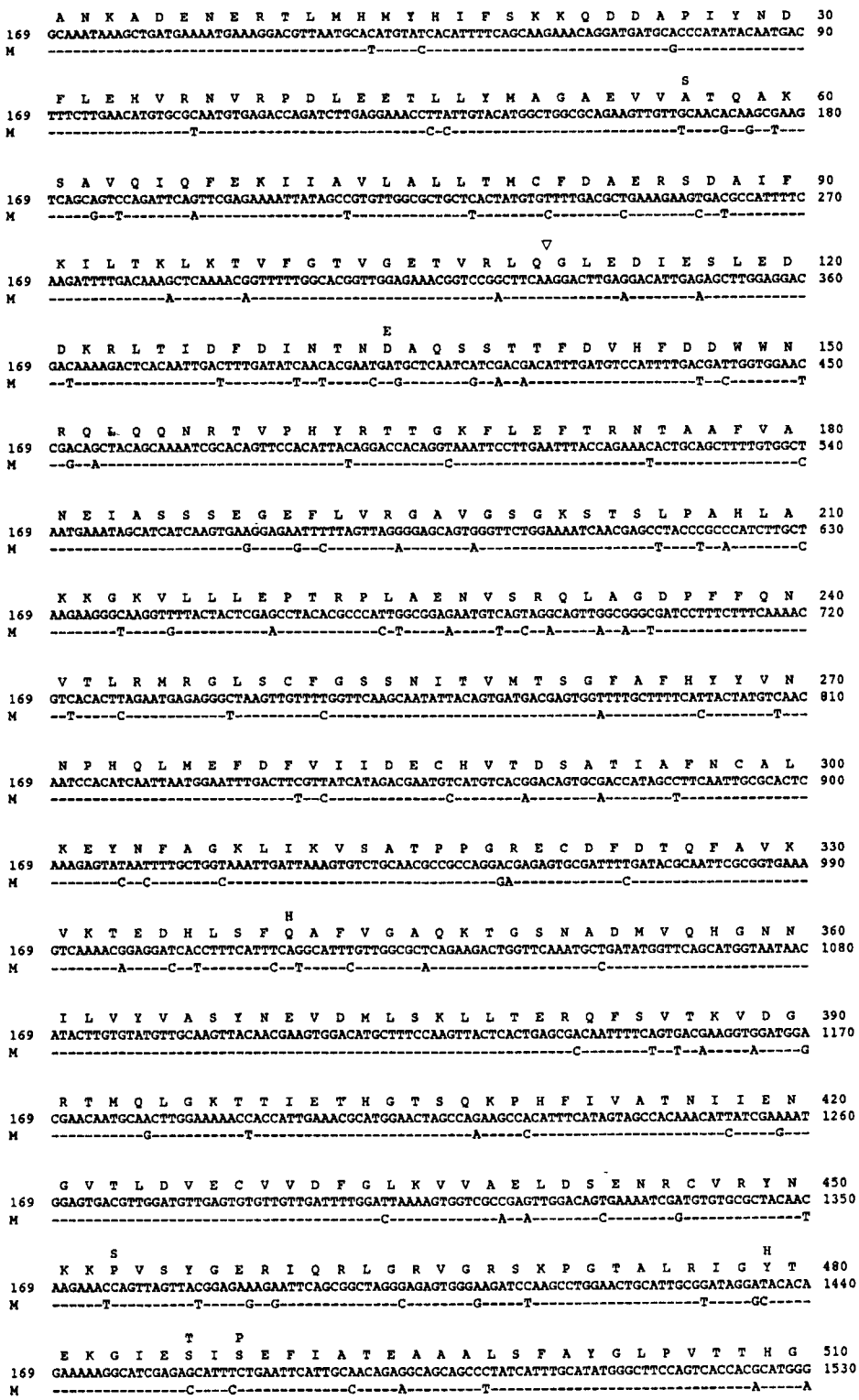
Abbreviations: aa = amino acid; CIP = cylindrical inclusion protein; CP = coat protein; ds = double-stranded; MLV = murine leukemia virus; nt = nucleotide; ORF = open reading frame; RT-PCR = reverse transcription-polymerase chain reaction; ZYMV = zucchini yellow mosaic virus



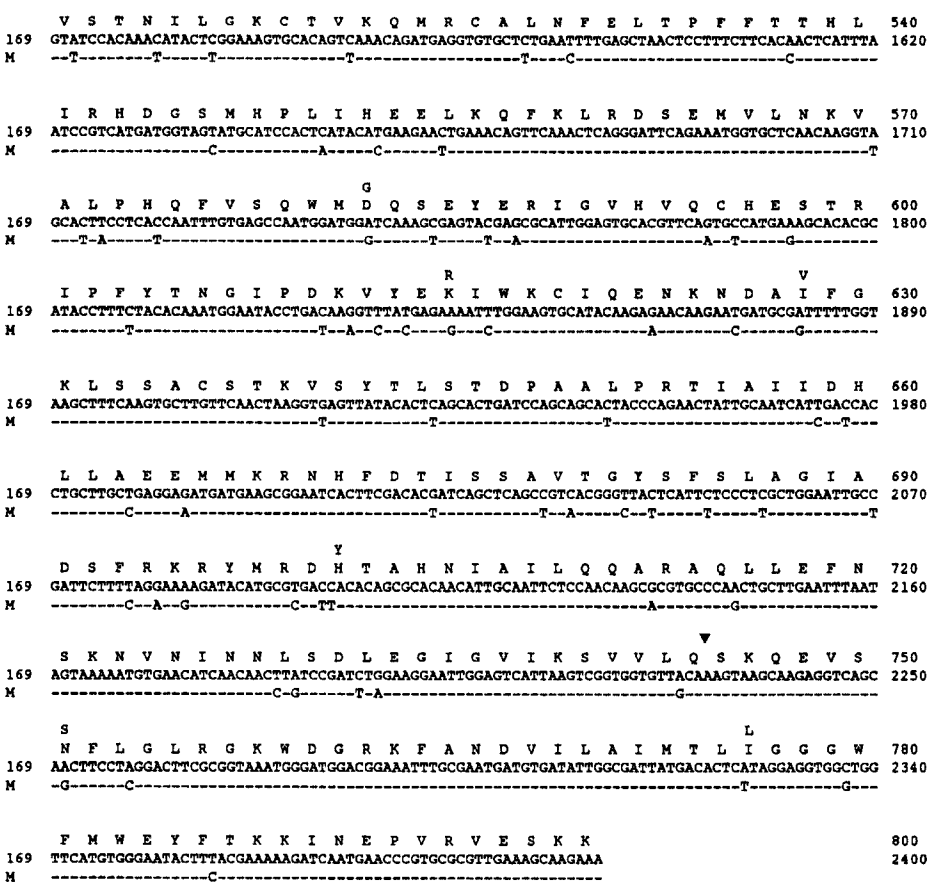


Fig. 1

Comparison of nucleotide and deduced amino acid sequences of CIP genes of ZYMV-169 and ZYMV-M cDNAs

Dashes show identical nucleotides in comparison to ZYMV-169. The deduced amino acid sequence of ZYMV-169 is shown above its nucleotide sequence. The different amino acids found in ZYMV-M are shown above the amino acids of ZYMV-169. The putative cleavage sites located between 6K1 and CIP, and between CIP and 6K2 genes are denoted by empty and full triangles, respectively.

lates and compared them with those of other reported ZYMV isolates by phylogenetic analysis.

The two Japanese ZYMV isolates (ZYMV-169 and ZYMV-M) (Kundu *et al.*, 1997) were propagated on *Cucurbita maxima* Duch. cv. Hokoseihi and purified according to Sako *et al.* (1980). The genomic RNAs of the ZYMV isolates were extracted by the procedure described by Rosner *et al.* (1983). The reverse transcription-polymerase chain reaction (RT-PCR) (Ohshima *et al.*, 1994) was employed for cDNA cloning of the CIP genes with some modifications. First-strand cDNAs were synthesized from ZYMV RNAs with minus strand oligonucleotide primers ZYMVCI2M (5'-GGGGCGGCCGCTTTCTTGCTTTCAACGCGC-3') and ZYMVCI4M (5'-GGGGCGGCCGCCAAGTATGTTATTACCAT GCTG-3') using murine leukemia virus (Moloney virus, MLV) reverse transcriptase (Gibco BRL). Double-stranded (ds) cDNAs were amplified from the first-strand ZYMV cDNAs using pairs of oligonucleotide primers ZYMVCI2P (5'-GGGGCGGCC GCCAGCATGGTAATAACATACTTG-3') and ZYMVCI2M (5'-GGGGCGGCCGCTTTCTTGCTTTCAACGCGC-3') or ZYMVCI4P (5'-GGGGCGGCCGCGCAAATAAAGCTGATGAAAATG-3') and ZYMVCI4M (5'-GGGGCGGCCGCCAAGTATGTTATTAC CATGCTG-3') by the PCR method (Saiki *et al.*, 1988). The oligonucleotide primers were designed on the basis of nucleotide sequence of ZYMV-Cal RNA (Balint *et al.*, 1994). The amplified ds cDNAs were digested with *Not*I endonuclease and inserted into the *Not*I restriction site of Bluescript II SK$^+$ plasmid (Stratagene). The recombinant plasmids were introduced into *Escherichia coli* XL1-Blue and then extracted by the boiling method (Holms and Quigley, 1981). The DNAs were sequenced using ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit. Primers based on the nucleotide sequences of Bluescript II SK$^+$ plasmid [RV (5'-AACAGCTATGACCATG-3') and KS (5'-CGAGGTC GACGGTATCG-3')] and the ZYMV isolates [ZYMVCI3P (5'-AAGCCTGGAACTGCATTGC-3'), ZYMVCI3M (5'-TGCAAT GTTGTGCGCTGTGT-3'), and ZYMVCI5P (5'-GCCATTT TCAAGATTTTGGACAA-3')] were used for sequencing. The nucleotide and deduced amino acid sequence analyses, similarity searches, and multiple alignments were carried out using the DNASIS program (Hitachi Software Engineering Co. Ltd. 1992). Phy-

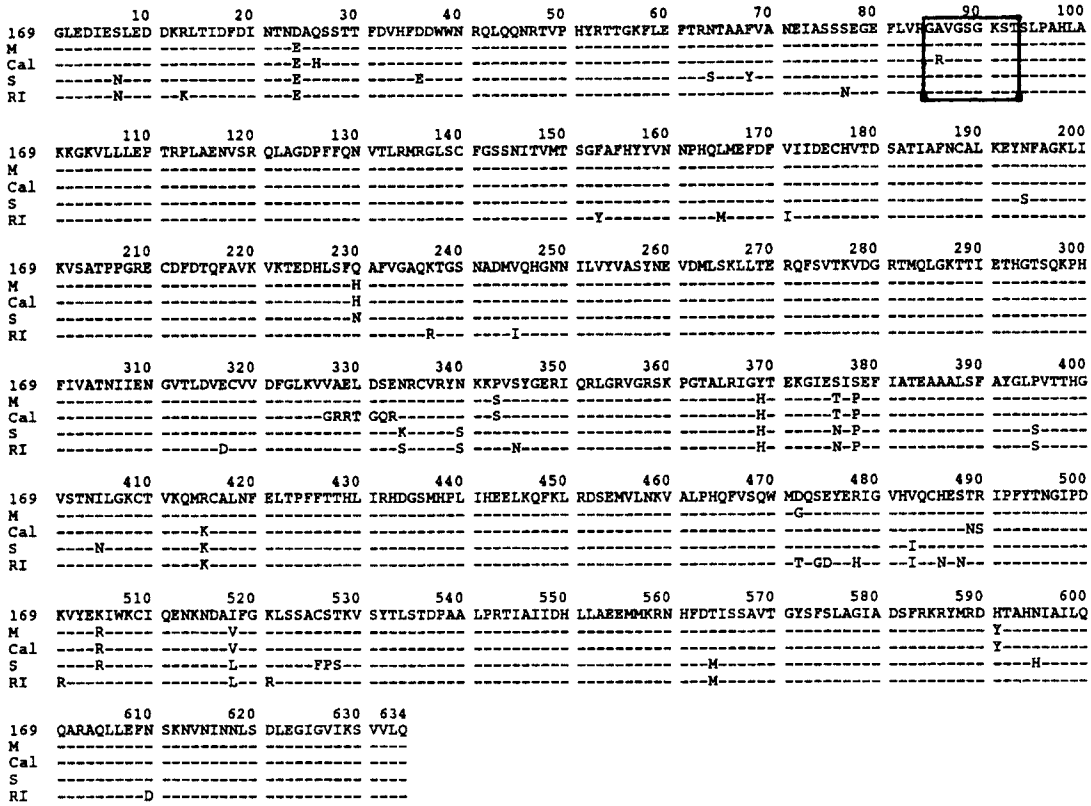


Fig. 2

Multiple alignment of deduced amino acid sequences of CIP genes of ZYMV isolates

Dashes show identical amino acids in comparison to ZYMV-169. The site of the so-called nucleotide binding motif is boxed (aa 85–93). The sources of CIP gene sequence data are Balint *et al.*, 1994 (ZYMV-Cal), Baker *et al.*, 1994 (ZYMV-RI), and Lee *et al.*, 1997 (ZYMV-S)

logenetic analysis of the deduced amino acid sequences of CIP genes was accomplished using the Protein Sequence Parsimony Method (PROTOPARS) of Phylogeny Inference Package (PHYLIP) as developed by Felsenstein (1993). For phylogenetic analysis, an ordinary strain (PVY-O) of potato virus Y (Ohshima *et al.*, 1993) was defined as outgroup. All data sets were subjected to bootstrap analysis by performing 100 replications using SEQBOOT in PHYLIP. The tree depicted was unrooted and both the horizontal and vertical branch lengths were arbitrary.

The nucleotide sequences of the ZYMV-169 and ZYMV-M CIP genes were determined from nine recombinant clones and will appear in the DDBJ, EMBL and GenBank nucleotide sequence data bases under Acc. Nos. AB020477 and AB020478, respectively. No differences were observed in overlapping regions of these clones. Nucleotide sequences of the cDNAs including a portion of P3 gene, 6K1 gene, the complete CIP gene, and a portion of 6K2 gene with deduced amino acid sequences are shown in Fig. 1. The protease cleavage site between the 6K1 and CIP genes was Q/G, and that between the CI and 6K2 genes was Q/S (Fig. 1). The CIP genes of ZYMV-169 and ZYMV-M were 1902 nucleotides in length, and encoded 634 amino acids (Fig. 1). The size of the CIP genes of ZYMV-169 and ZYMV-M was identical to that of other ZYMV isolates reported previously. Comparison of the nucleotide and deduced amino acid sequences of the CIP genes of ZYMV-169 and ZYMV-M revealed 91% and 98% similarities, respectively. A total of 163 nucleotide substitutions were present between the CIP genes of the two isolates and gave rise to 10 amino acid differences (Fig. 1). The nucleotide substitutions were present predominantly at the third base of the codon. A nucleotide binding motif with concensus sequence G/AXXXG KS/T (Laín *et al.*, 1989) was fully conserved in ZYMV-169 and ZYMV-M CIPs at aa 85–93 (Fig. 2). The nucleotide and deduced amino acid sequences of the CIP genes of Japanese ZYMV isolates were compared with those of previously reported ZYMV isolates. ZYMV–169 showed 82 – 91% nucleotide similarity and 95 – 96% amino acid similarity to other ZYMV isolates (Table 1) and had unique amino acids at positions 24, 369, 376, 378, and 518 (Fig. 2). On the other hand, ZYMV-M showed 81 – 97% nucleotide similarity and 95 – 97% amino acid similarity to other

Table 1. Percentage similarity of the nucleotide and deduced amino acid sequences of potyvirus CIP genes

	ZYMV-169	ZYMV-M	ZYMV-Cal	ZYMV-RI	ZYMV-S	PVY-O	PRSV-H	TuMV-J
ZYMV-169		91	90	82	86	61	61	64
ZYMV-M	98		97	81	85	62	61	63
ZYMV-Cal	96	97		82	85	62	61	63
ZYMV-RI	95	95	93		82	61	62	63
ZYMV-S	96	96	95	95		61	61	63
PVY-O	52	53	53	51	52		62	62
PRSV-H	55	55	54	54	55	54		62
TuMV-J	55	55	54	54	54	56	55	

The upper diagonal of this table shows the nucleotide similarities while the lower diagonal shows the amino acid similarities. The sources of the sequence data were: Balint *et al.*, 1994 (ZYMV-Cal), Baker *et al.*, 1994 (ZYMV-RI), Lee *et al.*, 1997 (ZYMV-S), Ohshima *et al.*, 1993 (PVY-O), Yeh *et al.*, 1992 (PRSV-H) and Ohshima *et al.*, 1996 (TuMV-J).

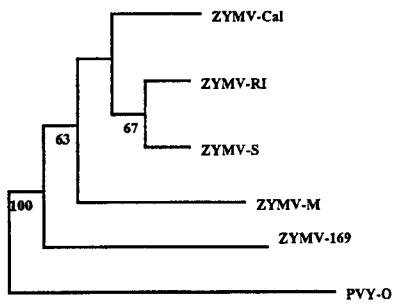


Fig. 3

Phylogenetic relationships among five ZYMV isolates based on multiple alignment of the deduced amino acid sequences of CIP genes

The ZYMV isolates are those shown in Fig. 2. The tree was obtained by the Protein Sequence Parsimony Method of PHYLIP with PVY-O (Ohshima *et al.*, 1993) defined as the outgroup. The tree shown is unrooted and both the vertical and horizontal branch lengths are arbitrary. Vaules at the nodes indicate the percentages of bootstrap analyses supporting the grouping. Bootstrap percentages below 50% are not shown.

ZYMV isolates (Table 1) and had unique amino acid at position 472 (Fig. 2). When the CIP genes of ZYMV and other potyviruses were compared, the nucleotide and amino acid sequence similarities ranged between 61 – 64%, and 51% – 56%, respectively (Table 1). These values are similar to those of other members of the *Potyvirus* genus (Shukla and Ward, 1988). A phylogenetic analysis derived from the deduced amino acid sequences of ZYMV CIP genes supported by low bootstrap values (less than 90%) showed that all the five ZYMV isolates clustered into one group (Fig. 3).

The above results indicate that the CIP genes of Japanese ZYMV isolates are very close to those of other ZYMV isolates (Figs. 2 and 3), although ZYMV-169 was shown to be the most distinct isolate compared to other ZYMV isolates when amino acid sequences of their CPs were analyzed by phylogenetic tree (Kundu *et al.*, 1997). Sequence similarities in different regions of the genomes of distinct potyviruses and strains showed that CIP is the most conserved protein after the RNA-dependent RNA polymerase (NIb gene) (Ward *et al.*, 1992).

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