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VIRAL AND VIROID DISEASE

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Nucleotide sequence of the coat protein gene of Mirafiori lettuce virus

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Abstract The coat protein (CP) gene of Mirafiori lettuce virus (MiLV), a tentative member of the genus *Ophiovirus* was isolated and sequenced. The established sequence consists of 1514 nucleotides including one open reading frame (ORF) with 1311 nucleotides that encodes 437 amino acids with a relative molecular mass 48543. When the ORF was expressed in *Escherichia coli*, the obtained protein was confirmed as CP by Western blotting using an antiserum against MiLV. Database searches showed that the CP gene of MiLV has a sequence similar to that of *Citrus psorosis virus* (CPsV), the type species of the genus *Ophiovirus*. The comparison between MiLV and CPsV CP genes revealed that the identities of the nucleotide and amino acid sequences were 46.5% and 30.9%, respectively.

Key words Mirafiori lettuce virus · *Ophiovirus* · Gene for coat protein

Introduction

Lettuce big-vein disease, first reported in the United States (Jagger and Chandler 1934), occurs in all major lettuce producing areas (*Lactuca sativa* L.) in the world and has become an important problem especially during the cooler periods of the year (Campbell 1996; Huijberts et al. 1990; Kuwata et al. 1983; Vetten et al. 1987). The main symptoms are vein bandings in the leaves of affected lettuce plants, with leaf distortion and ruffling. The economic importance of the disease is a result of the unsightliness of the foliage (which depresses the market value), delayed head formation, decreased head size, and a reduced number of harvestable plants (Zink and Grogan 1954). The causal agent of the disease has been thought to be a virus transmitted by the obligately parasitic soil-inhabiting fungus *Olpidium brassicae* (Campbell and Grogan 1963; Grogan et al. 1958; Tomlinson and Garrett 1962).

A rod-shaped particle of *Lettuce big-vein virus* (LBVV), first reported in Japan, was shown to be associated with the big-vein disease (Kuwata et al. 1983). The association of LBVV with big-vein disease was confirmed in Germany and Holland (Huijberts et al. 1990; Vetten et al. 1987), and LBVV was assumed to cause the disease. The rod-shaped particles were about 320–350 × 18 nm, with a coat protein (CP) of 48 kDa (Huijberts et al. 1990; Kuwata et al. 1983; Vetten et al. 1987). With the evidence on morphology, serology, vector relation, and other characteristics, LBVV was designated the type virus in an unassigned genus *Varicosavirus* in the 7th Report of the International Committee on Taxonomy of Viruses (ICTV) (Mayo 2000). However, reevaluation of etiology of big-vein disease has been necessary because a second virus, Mirafiori lettuce virus (MiLV), was found commonly in lettuce that exhibited big-vein symptoms (Natsuaki et al. in press; Roggero et al. 2000). Furthermore, because the lettuce infected with MiLV alone consistently developed big-vein symptoms regardless of the presence or absence of LBVV, MiLV was thought to be a main agent of big-vein disease (Lot et al. 2002).

MiLV is a multicomponent ssRNA virus with a CP of approximately 48 kDa (Roggero et al. 2000). MiLV particles contain three single-stranded RNAs about 8.5, 1.9 and 1.7 kb, respectively. MiLV is thought to be a member of the genus *Ophiovirus* (Roggero et al. 2000), which was also a

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currently established genus in the 7th ICTV report (Milne et al. 2000). Particles of ophioviruses are filamentous and circularized, and they have one CP of 43–50 kDa and three ssRNAs of 7.5–9.0, 1.6–1.8, and 1.5 kb, respectively. *Citrus psorosis virus* (CPsV), *Ranunculus white mottle virus* (RWMV), *Tulip mild mottle mosaic virus* (TMMMV), and MiLV belong to the genus (Milne et al. 2000; Roggero et al. 2002). The nucleotide sequences of RNA2 and RNA3 of CPsV have been determined (Barthe et al. 1998; Sánchez de la Torre et al. 2002), and RNA3 of CPsV has been reported to encode a CP (Barthe et al. 1998). The sequences of the other RNAs of ophioviruses are unknown.

Lettuce big-vein disease is the most serious soil-borne fungus-transmitted viral disease in leafy vegetables, not only in Japan (Kuwata et al. 1983) but also in Europe and the United States (Campbell 1996; Huijberts et al. 1990; Vetten et al. 1987). It is extremely difficult to control the disease because resting spores of the fungal vector carrying the pathogen internally survive under adverse conditions in the absence of the host, and highly resistant cultivars are lacking (Campbell 1962; Sánchez de al Torre 1998). One of the most promising ways to control the disease would be to transform MiLV-derived genes or genome fragments into lettuce. So far, there have been numerous attempts to generate virus resistance in transgenic plants through the expression of virus-derived genes or genome fragments, and many of the attempts have been successful (Lomonossoff 1995). In this paper, we report the nucleotide sequence of the MiLV CP gene, which will be useful for developing MiLV-resistant transgenic lettuce plants. Those plants are expected to have resistance to lettuce big-vein disease.

Materials and methods

Virus and antiserum

A virus isolate, designated MiLV-H, was obtained from a lettuce plant cv. Cisco showing typical big-vein symptoms and grown on infested soil collected from Hyogo Prefecture, Japan. After separating the virus from the infected lettuce to *Chenopodium quinoa* by mechanical inoculation according to Roggero et al. (2000), the virus was propagated in *C. quinoa*. The antiserum to MiLV was kindly provided by Dr. R. G. Milne.

Virus purification

The inoculated leaves of *C. quinoa* were harvested 10 days after inoculation. The purification method adopted was modified from one for purification of TMMMV previously described (Morikawa et al. 1995). Locally infected leaves of *C. quinoa* were homogenized with 50 mM Tris-HCl buffer pH 7.9 containing 5 mM Na-DIECA (Sodium *N*,*N*-Diethyldithiocarbamate Trihydrate), 0.1% (v/v) 2-mercaptoethanol, and 1 mM Na-EDTA. Clarification of crude extract with CCl_4 was omitted. Cs_2SO_4 equilibrium centrifugation was done instead of the final CsCl density gradient centrifugation.

Direct amino acid sequencing of MiLV CP-derived peptides

The N-terminal amino acid sequences of internal peptide fragments of the CP of MiLV-H were determined according to Hirano et al. (1991). Purified MiLV-H was dissolved in 1 × sodium dodecyl sulfate (SDS) gel loading buffer [50 mM Tris-HCl pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 2% (v/v) 2-mercaptoethanol] and subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970). After staining with Coomassie brilliant blue R-250, the protein corresponding to the CP of MiLV-H in the gels was excised, carboxymethylated, and digested by a lysylendopeptidase. The peptide fragments obtained were isolated using high-performance liquid chromatography (HPLC) with a Wakopak Navi C-22-5 reverse-sphere column (Wako, Osaka, Japan). The amino acid sequences of the peptide fragments were determined using an HP G1005A protein sequencing system (Hewlett-Packard, Palo Alto, CA, USA).

Cloning and sequencing of the CP gene of MiLV-H

Viral genomic RNAs were extracted from purified preparations with an equal volume of phenol/chloroform and subsequently precipitated with ethanol. After the ethanol precipitation, MiLV-H RNAs were resuspended in RNase-free water and then used immediately for complementary DNA (cDNA) synthesis by a random hexamer using a first-strand cDNA synthesis kit (Amersham-Pharmacia, Piscataway, NJ, USA).

The initial cloning of the MiLV-H genomic RNAs was achieved using degenerate primers dYK5 (5′-GARGGIG ARACIGCIAT-3′ corresponding to nucleotides 147 to 163, in which I and R indicate inosine and A or G, respectively) and dYK8 (5′-SWIACYTCIGTIGGIAR-3′ complementary to nucleotides 903 to 919, in which S, W, and Y indicate C or G, A or T, and C or T, respectively), which were designed from two amino acid sequences of the internal peptide fragments in the CP of MiLV-H (see Fig. 3, below). The cDNA was amplified by the polymerase chain reaction (PCR) in 50 µl of 1 × reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, and 200 µM each of the four dNTPs), 1.5 mM $MgCl_2$, 1 µM each of the primers, and 2.5 U *Taq* DNA polymerase (Promega, Madison, WI, USA). The PCR protocol consisted of 3 min at 94°C followed by 30 cycles of 30 s at 94°C, 1 min at 41°C, 1 min at 72°C, and finally an extension time of 10 min at 72°C.

Because the purified MiLV RNAs preparation contained positive-sense and negative-sense RNAs (Roggero et al. 2000), the upstream and downstream regions of the established sequence of the MiLV CP gene were verified using a 5′RACE system (Smart Race cDNA Amplification Kit; Clontech, Palo Alto, CA, USA) according to the protocol provided by the suppliers. After synthesizing first-strand cDNA primed by a random hexamer, the upstream region of the established sequence of the MiLV CP gene was

amplified using primer UPM supplied in the kit and a virus-specific primer, dYK7 (5′-CCYTGRTTRTAIACIARRCA RAA-3′ complementary to nucleotides 714 to 736). In the case of amplifying the downstream region, primer UPM and a virus-specific primer dYK12 (5′-TATCTGTCATCAG CAGATCC-3′ identical to nucleotides 837 to 856) were used. The PCR protocol consisted of 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 1 min at 56°C, 2 min at 72°C, and finally an extension time of 10 min at 72°C.

Amplified PCR products were separated by electrophoresis in a 1.5% agarose gel and isolated from the gel using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified PCR fragments were directly ligated into plasmid pGEM-T Easy Vector (Promega). At least three clones of each PCR fragment were sequenced in both directions using an automated ABI Prism 377 DNA sequencer with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA, USA). Nucleotide sequence data were compiled and analyzed with Genetyx-Win version 5.1 (Software Development, Tokyo, Japan). The amino acid sequence of the MiLV CP was compared with the DDBJ database using the TFASTA program (Pearson and Lipman 1988).

Expression of the MiLV CP in *Escherichia coli* and Western blot analysis

A clone containing the full-length CP gene of MiLV-H was amplified by PCR using primers dYK17 (5′-cgcggatcc TCAGGAGTATACAAGGTTTCC-3′ in which uppercase letters correspond to nucleotides 90 to 110; the *Bam* HI site is underlined) and dYK18 (5′-gcgctgcagTCATTTCTTTC CGTAAGCTGT-3′ in which the uppercase letters are complementary to nucleotides 1380 to 1400; the *Pst* I site is underlined). After digestion with *Bam* HI and *Pst* I, the PCR product was ligated into *Bam*HI and *Pst*I sites of the pQE-80L expression vector (Qiagen). The resulting plasmid was used to transform *Escherichia coli* strain JM 109 (Toyobo, Osaka, Japan). For expression, a fresh single colony was grown at 37°C in LB medium containing ampicillin (100 μg/ml). When the optical density at 600 nm (OD_{600}) of the suspension reached 0.6, the expression was induced by 1 mM IPTG. Aliquots of induced cells were concentrated by centrifugation, disrupted by boiling in 2 × SDS gel loading buffer, and electrophoresed on 10% SDS-polyacrylamide gels. Western blotting analysis was then conducted according to a standard procedure (Gallagher 1993). The gels were transferred to nitrocellulose membranes using semidry systems. The antiserum against MiLV (a gift of Dr. Milne) was used for the primary antibody, and alkaline phosphatase-conjugated goat antibody (Bio-Rad, Hercules, CA, USA) was used for the secondary antibody. Finally, the membranes were developed using BCIP/NBT visualization solution.

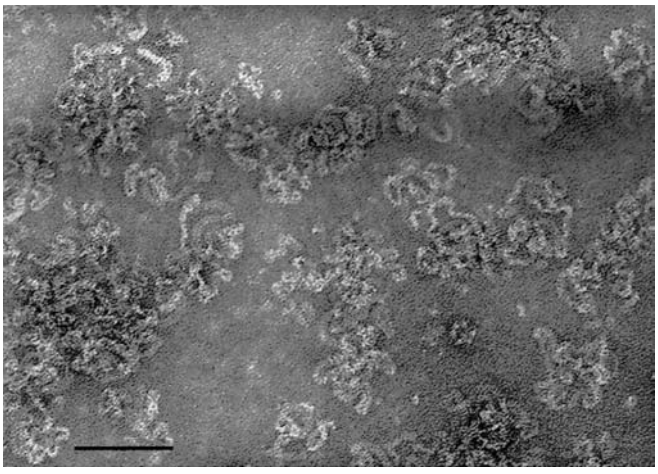

Fig. 1. Electron micrograph of MiLV particles purified from *Chenopdium quinoa*, negatively stained in uranyl acetate. *Bar* 100 nm

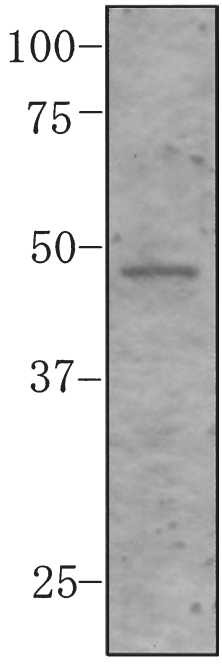

Fig. 2. Analysis by 10% Sodium dodecyl sulfate-polyaerylamide gel electrophoresis of the protein associated with purified MiLV particles. Molecular weight markers (in kilodaltons) are indicated at the left

Results and discussion

Purification of MiLV-H virions

After Cs_2SO_4 equilibrium centrifugation, only one light-scattering band was usually observed (data not shown). Electron microscopy revealed large numbers of ophiovirus-like particles in purified preparations (Fig. 1). The particles were the same as those described by Roggero et al. (2000) and Lot et al. (2002). No varicosavirus-like particles were detected. When the purified MiLV-H preparation was analyzed for purity by SDS-PAGE, only one protein of about 48 kDa was detected (Fig. 2).

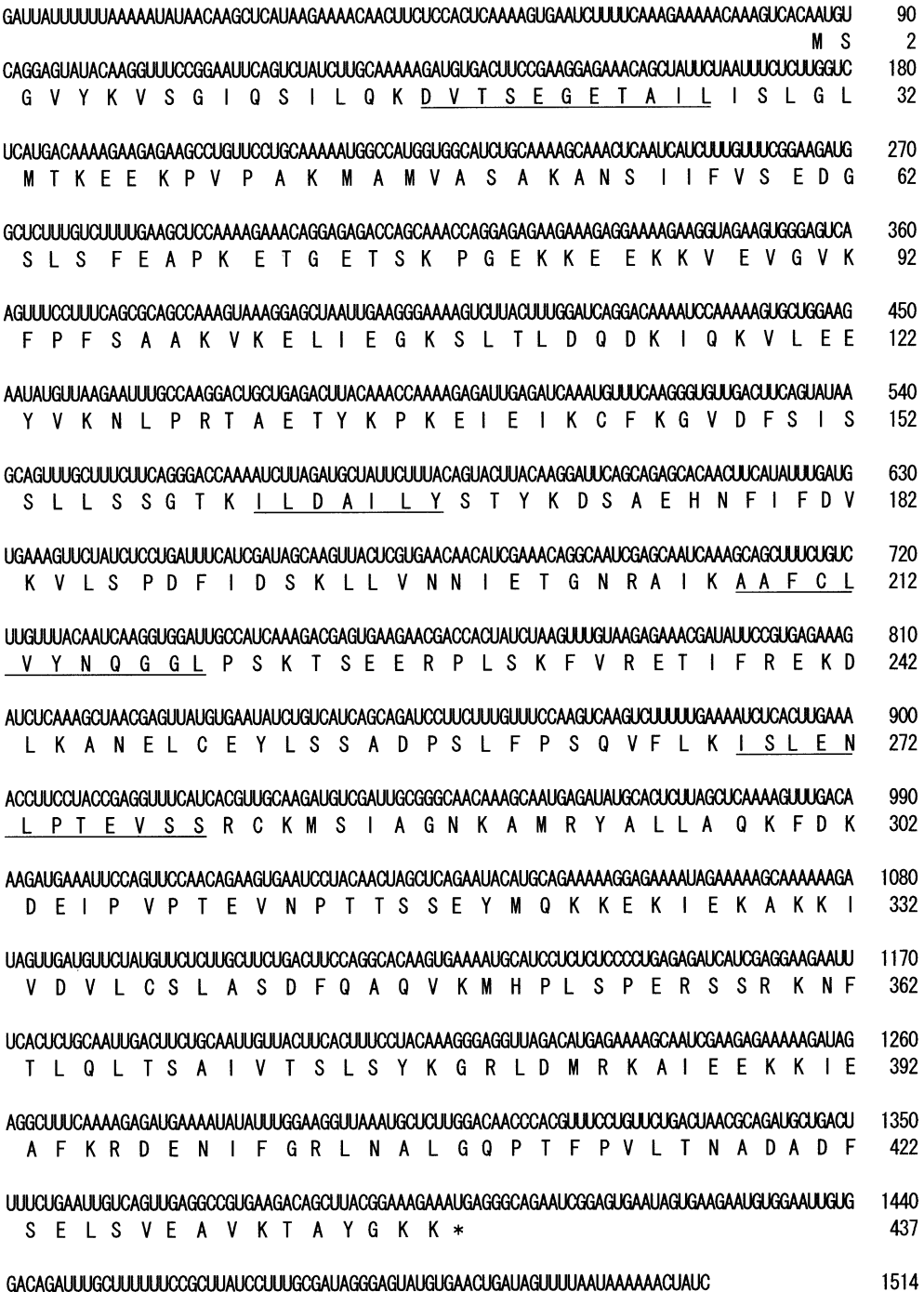

Fig. 3. Positive-sense nucleotide sequence and the deduced amino acid sequence of the MiLV CP gene. Determined amino acid sequences of the internal peptide fragments in the MiLV CP are underlined. A stop codon is indicated with an *asterisk*

Sequence of the CP gene of MiLV-H

The N-terminal amino acid sequences of four internal peptide fragments of the MiLV CP were determined after digesting the CP with lysylendopeptidase (Fig. 3). Four degenerate primers were designed on the basis of these sequences. Reverse transcription (RT)-PCR using the combination of degenerate primers dYK5 and dYK8, designed from DVTSEGETAIL and ISLENLPTEVSS, respectively, gave only a single product of approximately 0.75 kb, and the sequence was determined.

Because MiLV RNA preparations contained positive-sense and negative-sense RNAs (Roggero et al. 2000), both upstream and downstream regions of the established sequence were determined by a 5′RACE system. A total of 10 clones derived from the 5′RACE-generated cDNA were amplified using a primer UPM and a virus-specific primer dYK7, and the sequence of a fragment of 736 nucleotides was determined. The 10 clones showed variations in size at their 5′ end. The longest sequence (5′-GAUUAUUUU UUAA. . . . -3′) was found in 3 of the 10 clones. The other seven clones had deletions of 21 (three clones), 22 (two

clones), and 24 (two clones) nucleotide residues from the 5′ end. The sequence of a fragment of 678 nucleotides of the downstream region was determined using the primer UPM and the virus-specific primer dYK12. The variations of the 11 5′RACE-generated cDNA clones in their 3′ end sequence were also observed. Seven of the eleven clones showed the longest sequence (5′-. . . AUAAAAAACU AUC-3′). The other four clones had deletions of 1 (one clone), 13 (two clones), and 16 (one clone) nucleotide residues from the 3′ end. Whether these deletions were due to true variability or artifacts is not known. Subsequently, the sequence of the entire 1.5 kb region was confirmed by recloning the MiLV genomic RNAs using virus-specific primers and sequencing at least three independent clones.

Complementarity between the 5′ and 3′ termini is a common feature of the genomes of negative-strand RNA viruses (Tordo et al. 1992). In the case of the established sequence from MiLV-H, the complementarity was observed between the 5′ and 3′ ends in the negative strand (Fig. 4). Because U pairs not only with A but also with G in the RNA secondary structure (Varani and McClain 2000), 11 of 12 nucleotides at each end can be paired. This result suggests that the 1514 nucleotides sequence could correspond to the one of three separated MiLV RNA segments.

The 1514 nucleotides contained one open reading frame (ORF) beginning with the first AUG codon at positions 87 to 89 (Fig. 3). No other ORFs of significant size were found on either of the strands. One amino acid sequence obtained by internal peptide fragments of the MiLV CP was DVTSEGETAIL, and nucleotide position 135 to 167 encoded the same amino acid sequence. There was only one AUG sequence between positions 1 and 135. Furthermore, the sequence surrounding the AUG sequence CACAAUGUC was close to the most favored plant initiation consensus sequence for plants, AACAAUGGC (Lütcke et al. 1987). These results suggest that the AUG sequence at positions 87 to 89 is the authentic translation initiation site.

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5′ - GAUAGUUUUUUAUUAAAACUAUCAGUUCACAUACU·····
     ||| :|||||||  || |  |: ||:||| |
3′ - CUAAUAAAAAAUUUUUAUAUUGUUCGAGUAUUCUU·····
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Fig. 4. Complementarity between the 5′ and 3′ ends of the established nucleotide sequence (negative-sense). The *upright lines* and *colons* indicate Watson-Crick basepairing and G × U wobble basepairing, respectively

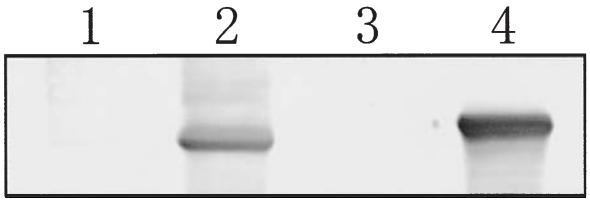

Fig. 5. Western blot analysis of MiLV coat protein expressed in *Escherichia coli*. Proteins were extracted from healthy leaves of *C. quinoa* (lane *1*), MiLV-H infected leaves of *Chenopodium quinoa* (lane *2*), *E. coli* transformed with the vector plasmid alone (lane *3*), and *E. coli* expressing the CP gene of MiLV-H (lane *4*). Blots were developed using the antiserum against MiLV

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MiLV   MSGVYKVSGIQSILQKDVTSEG-----ETAILISLGLMTKEEKPVPAKMAMVASAKANSI  55
       ||   ||| : .   |  :.:        |::| : |:: :     . . | :|. . :.
CPsV   MSIPIKVSQLIDAKAKWESDKENCPAEITTLLKTYGVLRETGALSSKSTAYLAGLSKDKR  60

MiLV   IFVSEDGSLSFEAPKETGETSKPGEKKEEKKVEVGVKFPFSAAKVKELIEGKSLTLDQDK  115
       ::::::|  .|:  ::        |||   . . .     .    :|: :| ::: :|| |
CPsV   VILTDEGLAEFKNVEN---PHDQGEKPTFSLASSSNIKITDIEVIKQKMEQETFQIDQLK  117

MiLV   IQKVLEEYVKNLPRTAETYKPKEIEIKCFKGVDFSISSLLSSGTKILDAILYSTYKDSAE  175
       ::: :|:::|.:.   |:|.  || || | . | .:. |:::|||||.::| : |..::
CPsV   LKEQIENFIKTVTLDDESYTEGEIVIKHFGNPDAELNMLVTAGTKILDGLVYVSMKGDTK  177

MiLV   HNFIFDVKVLSPDFIDSKLLVNNIETGNRAIKAAFCLVYNQGGLPSKTSEERPLSKFVRE  235
          :  .|: . | : .. :|:||...:|||:||| ||:.||.||.|:.::| :.:||:.
CPsV   S--LNLFKMEQVDGVCDSDIVKNIRVAKRAIQAAFVLVFTQGSLPGKADDKRKVPEFVKS  235

MiLV   TIFREKDLKANELCEYLSSADPSLFPSQVFLKISLENLPTEVSSRCKMSIAGNKAMRYAL  295
       .::   |:. .::.| || | .. ||::|||||.::|||: |.||||:.||||:::|||
CPsV   KLYDG-DVSLSQISEELSHAPTKKFPARVFLKIDIDNLPSAVCSRCKLNIAGNRSVRYAS  294

MiLV   LAQKFD-KDEIPVPTEVNPTTSSEYMQKKEKIEKAKKIVDVLCSLASDFQAQVKMHPLSP  354
       :|..|: |:::. . . ..| :    ::.::|||||  | |.| :: .::: | ::|||||
CPsV   FASSFQTKQKLSPAVGATPESLMPLLETNQKIEKAIAIRDFLKTMEGQWKNQKRLHPLSD  354

MiLV   ERSSRKNFTLQLTSAIVTSLSYKGRLDMRKAIEEKKIEAFKRDENIFGRLNALGQPTFPV  414
       |:.: |||||:||.||: ||: .|| || : | ..: :.|:.|.|:||  :.    |:.|
CPsV   EKPTIKNFTLKLTCAIIYSLTPDGRTDMAERIISDNNKGFQNDRNFFGDGEGP-IRTWSV  413

MiLV   LTNADADFSELSVEAVKTAYGKK                                       437
       ||:.:||||:::|:.:|  :|
CPsV   LTKPEADFSNITVDGLKGIFGVAATM                                    439
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Fig. 6. Comparisons of the amino acid sequence of the MiLV CP with that of CPsV. The *upright lines*, *colons*, and *periods* indicate identical amino acids, functionally conserved amino acids, and amino acids with low similarity, respectively. *Dashes* denote a gap

This ORF encoded a 437-amino-acid protein with a calculated M_r of 48543, which corresponded to the estimate of 48 kDa obtained by SDS-PAGE (Fig. 2). All amino acid sequences of four internal peptide fragments derived from purified MiLV-H virions existed in the deduced amino acid sequence from the ORF. When the ORF was expressed in *E. coli*, a protein similar in size to the CP of MiLV-H was detected using the antiserum against MiLV (Fig. 5). The results described above indicate that the established sequence originates from the viral RNA segment coding the MiLV CP gene.

FASTA analysis using the deduced amino acid sequence of the MiLV CP gene revealed significant similarity to that of the CPsV CP (Fig. 6). No significant similarities were found to other known viral sequences. The length of the amino acid sequence of the MiLV CP was two amino acid residues shorter than that of the CPsV CP (Barthe et al. 1998). A comparison of MiLV and CPsV CPs showed that the identities of the nucleotide and amino acid sequences were 46.5% and 30.9%, respectively. It has been reported that CPsV CP is encoded by RNA 3, and that RNA 3 consists of approximately 1500 nucleotides (Barthe et al. 1998; Sánchez de la Torre 1998). Judging from these reports, the 1514 nucleotide sequence determined in this study might be the sequence of RNA 3 of MiLV.

The successful cloning, sequencing, and bacterial expression of the MiLV CP gene will provide tools for studying the relations of MiLV with other ophioviruses. Furthermore, the availability of cDNA clones covering the whole MiLV CP gene will allow research on the development of pathogen-mediated resistance with transgenic lettuce expressing the MiLV CP gene to control MiLV. Such resistance has been obtained in tobacco to *Tomato spotted wilt virus*, which is the type member of the genus *Tospovirus* and contains segmented negative-stranded RNAs, by transforming plants with the gene harboring the viral nucleocapsid protein (Gielen et al. 1991).

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