Cloning and expression of turnip mosaic virus isolated from Hangzhou

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Abstract

One virulent isolate of turnip mosaic virus was isolated from infected *Brassica rape* in Hangzhou suburbs. The large scale preparation of the virus was purified by sucrose gradient centrifugation. The purified virus particles observed by electronic microscope were about 740nm × 12nm. The cDNA was synthesized with oligo$(dT)_{15}$ and PCR was carried out with two mutagenesis primers flanking the coat protein gene. One specific DNA segment was amplified. It was cloned into pUC18. The sequence of the coat protein gene was 867 nucleotides long determined by Sanger dideoxy sequencing. It was compared with four reported TuMV coat protein gene sequences. The homologues of nucleotide sequences between them were from 89.5% to 97.6% and the homologues of amino acid sequences were from 94.5% to 97.9%. The percentage of AT and GC, the nucleotide frequencies and the position frequencies were calculated. The codon usage of coat protein was analyzed. The coat protein gene was subcloned into the prokaryotic expression vector pKK233-2. The *E. coli* containing the expression vector with the insertion fragment was induced by IPTG. It was proved that the coat protein gene was expressed in *E. coli* by Western blotting.

Key words: TuMV; Coat protein gene; Expression

Introduction

Turnip mosaic virus, a member of the potyvirus group causes heavy yield losses in *Brassica* crops all over the world. The particle of virus is 740nm × 12nm containing a monopartite plus- sense, single stranded RNA genome of approximate 9800 nucleotides. The sequence encodes a polyprotein of 360 KDa more than 8 functional proteins. The virus infects mainly *Brassica* genus and is one of the most important viruses for vegetables. It is sap-transmissible and spread by over 40 species of aphids in a non-persistent manner (Shattuck et al., 1989).

Materials and methods

1. Virus and the RNA purification Turnip mosaic virus was isolated from Hangzhou suburbs in 1990 which was with strong infectivity to *Brassica rape* (Xu Ping et al. , 1994). The virus was propagated in *Brassica rape*. Virus purification procedures based as described by Nicolas and Laliberte (1991) except one cycle PEG differential speed centrifugation then purification with 10%— 40% sucrose gradient centrifugation. RNA was prepared as described (Nicolas and Laliberte, 1991).

2. Complementary DNA (cDNA) synthesis and polymerase chain reaction(PCR) First strand cDNA was synthesized by oligo$(dT)_{15}$ priming and by random priming of turnip mosaic virus RNA. The second strand cDNA was synthesized by standard method using RNase H and *E. coli* DNA polymerase I (Li Debao and Xu Ping, 1994). The efficiency of cDNA synthesis was determined by agarose gel electrophoresis to check the incorporation of [$\alpha-^{32}$P] dATP in the cDNA.

Double strand cDNAs were used as templates for PCR. Two mutagenesis primers were synthesized according to reported turnip mosaic virus coat protein genes. The 5' end primer was 5'—GCGTCGACCATGGCAGGTGAAACGCTTG—3' which contained additional initiation codon ATG and a SalI and a NcoI restriction enzyme sites. The 3' end primer was 5'-TGTCGACTTTATTCCCGGGTCATAACCCCTGAACGCC - 3' which contained a additional consensus transcription terminal sequence TTATTT and a SmaI and a SalI restriction enzyme sites. PCR was carried out using Perkin-Elmer Cetus DNA Thermal Cycler. The reaction was conduced in a volume 100 μl (10mmol/L Tris-HCl pH 8. 3, 50 mmol/L KCl, 2. 0 mmol/L $MgCl_2$, 20μmol/L each of four dNTPs and 2. 5 units of Taq DNA polymerase). The reactions were performed under the conditions: denaturation templates at 94℃ for 5 min, then with 30 cycles: denaturation at 94℃ for 45s, annealing at 48℃ for 2min, and extension at 72℃ for 3min, the extension at 72℃ for 20min in last cycle.

3. DNA manipulation The PCR product was visualized on a 1. 0% agarose gel and the product ends were made blunt by Klenow fragment and T4 DNA polymerase. Then they were cloned into pUC18 digested with SmaI and transformed into JM109. Minipreparation plasmid extractions were made by the alkaline lysis method and large scale plasmid preparations were made by CsCl-ethidium bromide gradient equivalent centrifugation (Li Debao and Xu Ping, 1994). Restriction endonuclease were purchased from Boehringer Mannheim.

4. DNA sequencing Several clones were analyzed by restriction mapping and the insert fragment was subcloned and sequenced by the dideoxynucleotide chain termination method (Sanger, 1977) using DNA sequencing kit with T7 DNA polymerase and M13/pUC universal primers from United States Biochemical Corporation. Primer extensions were carried out by using[$\alpha-^{35}$S]dATP and T7 DNA polymerase. The radiolablled products were loaded onto a

6% sequencing polyacrylamide gel and electrophoresis and autoradiograph as described by Ausubel et al. (1992). The sequences were analyzed by the NASA DNA Sequence Program and compared with turnip mosaic virus coat protein gene sequences reported in GenBank.

5. Immunological analysis The insert fragment was subcloned into prokaryotic expression vector pKK233—2. The *E. coli* strain JM105 containing the pKK233—2 with coat protein gene was cultured in 5ml Luria broth medium containing 100μg/ml ampicillin. When the cultural density reached OD_{600} to 0.5, IPTG was added to final concentration 1mmol/L and cultured continually for 6hrs to induce expression. The cells were harvested by centrifugation at 12000g. The precipitated cells were resuspended in 500μl ice cold 50mmol/L Tris—HCl (pH 7.4) and centrifuged at 12000g. The pellets were resuspended in 25μl H_2O and 25μl 2×SDS gel loading buffer and boiled for 5 min. The Western blotting was made by SDS—PAGE and detected by antibody as described by Sambrook et al., (1989). The primary antibody used in the reactions was polyclonal rabbit serium against turnip mosaic virus Hangzhou isolate.

Result

1. cDNA synthesis and coat protein gene cloning Virus was isolated and purified by sucrose gradient centrifugation. The pure viral particles were about 740nm × 12nm estimated by electronic microscope after phosphotungstic acid staining. No other viral particles were observed. Viral RNA was isolated from the purified turnip mosaic virus preparation. A pilot cDNA synthesis reaction labelled with [α —^{32}P]dATP was carried out and analyzed by electrophoresis on a alkaline agarose gel. The cDNA population were in different sizes over 0.5kb to 6.0kb.

The PCR amplification were performed as described in materials and methods and one specific band with perspective size about 0.86kb was obtained. Both cDNA synthesizedwith oligo$(dT)_{15}$ primer and with random primer obtained the same specific band in the amplifications. The DNA was treated by Klenow fragment and T4 DNA polymerase and ligated with pUC18 digested with SmaI. Following transformation, minipreparation plasmid isolations of approximately 40 white colonies growing on X—gal, IPTG and ampicillin selection plates were prepared. 12 colonies contained plasmids with 0.86kb coat protein gene insert. The restriction mapping of a recombinant DNA (termed pTuMVX) was made by single and double restriction enzyme digestion (Fig. 1).

2. Subcloning and DNA sequencing There were a EcoRI site and a XbaI site in the coat protein gene showed in Fig. 1 which were used to digest and subclone the insert for sequencing. The subclones were analyzed by plasmid minipreparation and digestion. The subclones containing inserts 0.48kb and 0.38kb were obtained. Large scale plasmid preparations by CsCl— ethidium bromide equivalent gradient centrifugation were prepared from the sub-

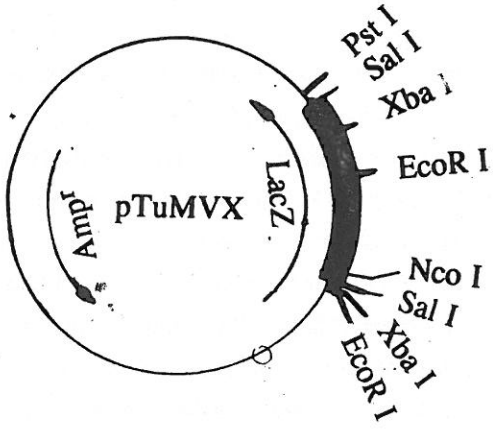

Fig. 1 The restriction map of pTuMVX

clones. The 867 nucloetides of coat protein gene were obtained entirely by forward and reverse M13/pUC universal primers. Update there are 4 turnip mosaic virus coat protein sequences reported in GenBank, TuMVK (Kong LJ et al., 1990), TuMVG (Greenland, AJ, et al., 1992 unpublished), TuMVN (Nicolas, O. and Laliberte, 1991) and TuMVP (Park, Y. et al., 1993 unpublished). The sequence was compared (Fig. 2) and shared high homology with them. The nucleotide sequence homologues of TuMVK, TuMVG, TuMVN and TuMVP were 89.5%, 97.6%, 96.7% and 91.7% respectively. The turnip mosaic virus coat protein gene coded for 289 amino acids. The amino acid sequence of TuMVK, TuMVG, TuMVN and TuMVP were 94.5%, 97.9%, 97. 9% and 97.9% of homologues respectively (Fig. 3).

The sequence was analyzed by NASA DNA Sequence Program. The AT frequency was 52.13% and GC frequency was 47.87%. The frequencies of four nucleotides in the sense DNA were A 33.10%, C 20.99%, G 26. 87% and T 19.03%. A was much high than T. The position frequencies of four nucleotides in open reading frame codon was showed in Table 1. It was showed that A was with high frequency in second position of codon and T was with low frequency in first position of codon. The codon usage of the turnip mosaic virus coat protein gene was more similar with dicotyls than mammalian (data not showed).

Table 1 Position frequencies of four nucloetides in open reading frame

position	A	C	G	T
1	96	58	98	37
2	112	57	50	70
3	79	67	85	58

3. Immunodetection of viral coat protein expressed in *E. coli* The TuMV antibody was used with goat anti — rabbit (GAR) antibody labelled with alkaline phosphatase(AP) as the detection system. The bacteria containing pKK233—2 with or without the coat protein gene were induced by IPTG and lysised by boiling for Western blotting.

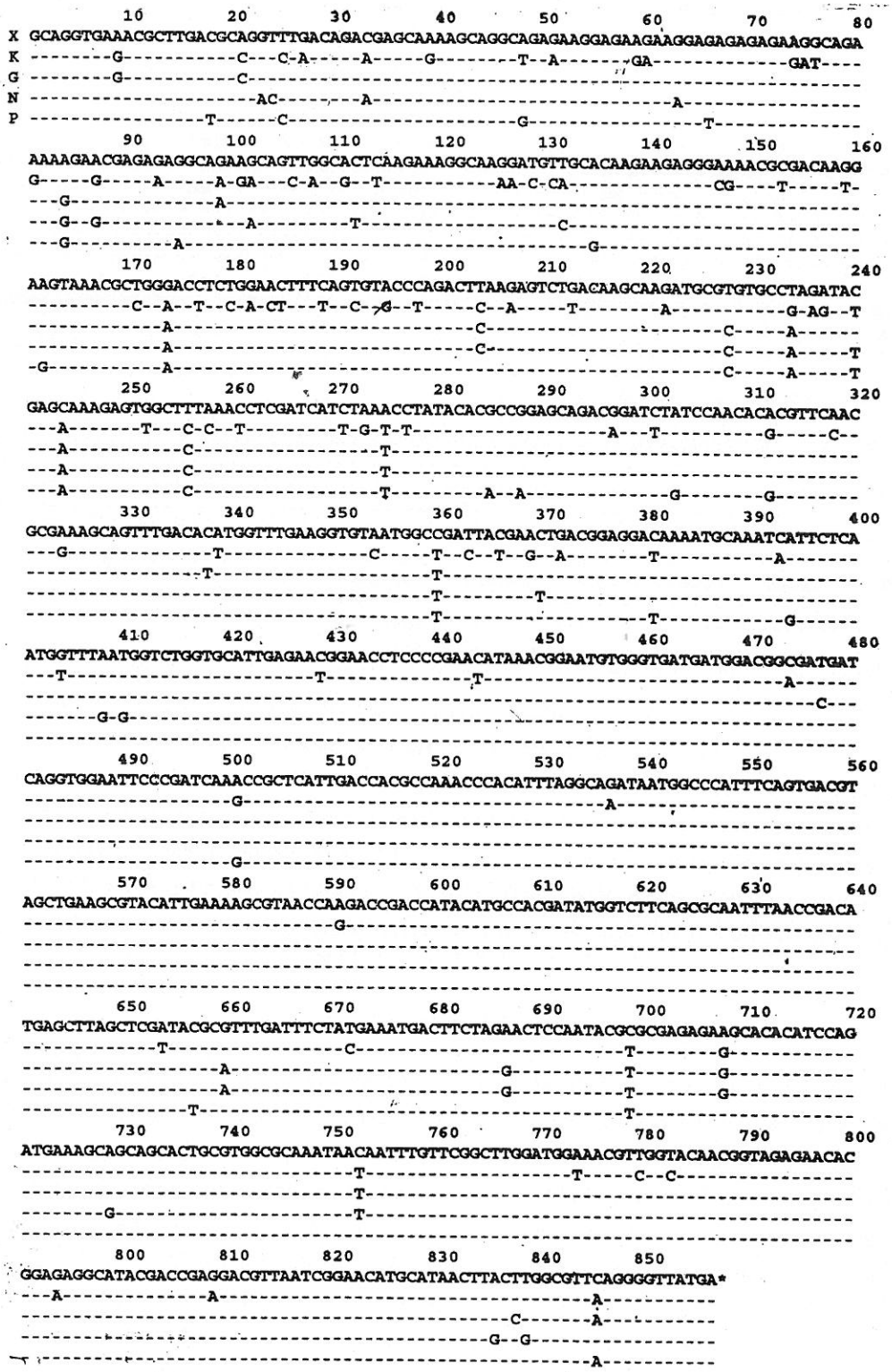

Fig. 2 Comparison of DNA sequences of TuMV coat proteins

X: TuMVX, K: TuMVK, G: TuMVG, N: TuMVN, P: TuMVP

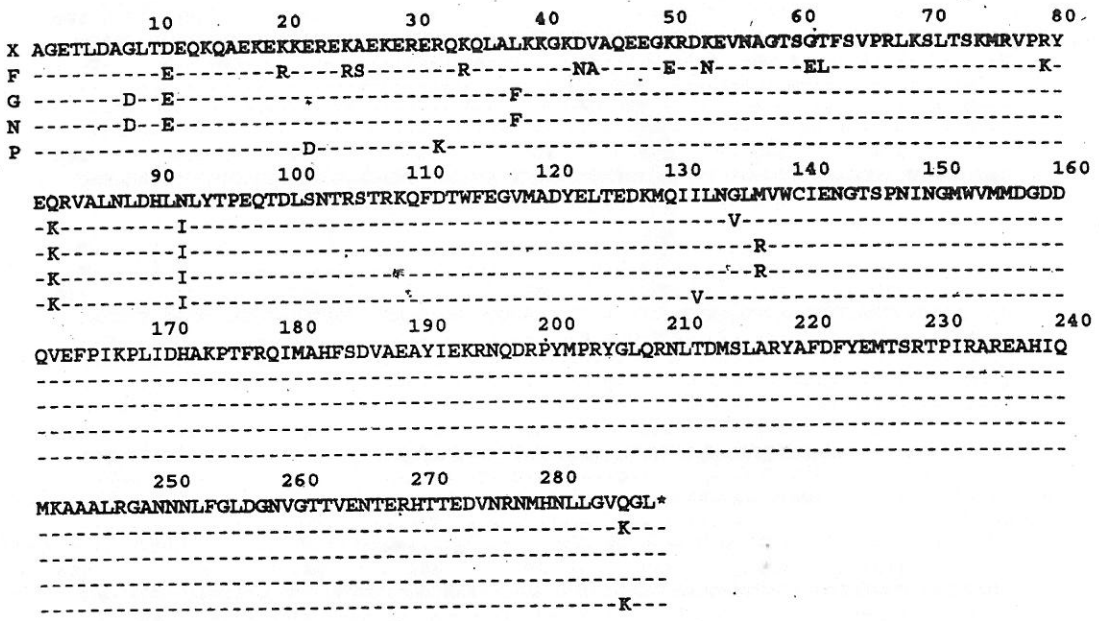

Fig. 3 Comparison of amino acid sequences of TuMV coat proteins
X: TuMVX, K: TuMVK, G: TuMVG, N: TuMVN, P: TuMVP

After bacterial lysates were transferred from SDS— PAGE to nitrocellulose membrane by electronic transferring, the viral antiserum was used to probe the coat protein position. Positive reaction was observed with one specific band of 32 KDa protein indicating the expression of virus coat protein in the bacterial cells (Fig. 4). The antiserum also reacted with native turnip mosaic virus but did not react with bacterial lysate containing nonrecombinant plasmid.

Discussion

In this report we described the cloning and sequencing of the coat protein of turnip mosaic virus Hangzhou isolate and expression in *E. coli*. It is a part of research on making transgenic plant resistant to turnip mosaic virus in our laboratory. This virus isolate was chosen because of its high virulence to *Brassica rape*. In order to modify the coat protein coding region for expression in prokaryotic and eukaryotic organisms we designed two mutagenesis primers to introduce necessary components. We added ATG codon and a consensus sequence 5'—ACCATGG—3' for initiation of translation by eukaryotic ribosomes (Kozak 1989) in 5' terminal primer and added a polyadenylation sequence AATAAA in 3' terminal primer. Analysis of the turnip mosaic virus Hangzhou isolate coat protein gene indicates that there is a little difference among the isolate and other isolates. The homologues of the amino acid sequence are different in either ends. The carboxy—terminal halves are more similar

with each other than the amino — terminal halves. Some researchers classified different turnip mosaic virus isolates by biological identification and monoclonal antibodies(Liu Xuping et al. 1990). The different isolates might be with different virulence to plants. Comparison of these different isolates will lead to locate the virulent regions and provide the basement for disease resistant research.

The use of PCR with Taq DNA polymerase is a powerful method in molecular cloning. Because the method has advantage in diagnosis, mutagenesis, sequencing and cloning it has been used in all fields of biological researches recently. Since the Taq DNA polymerase has not editing function and the products always contain mismatched sequences in amplification, it should be cautious to get concludes in sequence analysis. The sequences should be proved by clones from independent amplification products or by direct sequencing of amplification products.

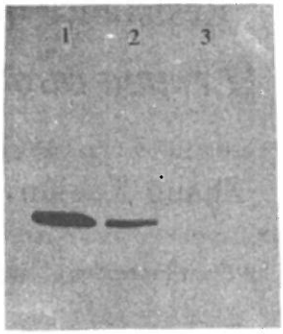

Fig. 4 Expression of turnip mosaic virus coat protein gene in *E. coli*
Lane 1: Purified virus; Lane 2: JM105 containing pKK233－2 with inserted coat protein gene; Lane 3: JM105 containing pKK233－2 only

Reference

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