Nucleotide Sequence of the 3'-Terminal Region of Bean Yellow Mosaic Virus RNA and Resistance to Viral Infection in Transgenic Nicotiana benthamiana Expressing Its Coat Protein Gene*

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Abstract

The sequence of 4,306 nucleotides from the 3'-poly(A) tract of bean yellow mosaic virus (BYMV) RNA has been determined. This sequence contained a single, long open reading frame (ORF), that when compared with amino acid sequences of other potyviruses was expected to encode carboxyl terminus of CI, 6K, Nla, Nlb and coat protein (CP). The CP-coding sequence was predicted to be 819 nucleotides in length, encoding a protein of 273 amino acids. To produce transgenic plants resistant to viral infections, the BYMV CP gene was fused to a high expression promoter cassette containing a duplicated enhancer sequence of cauliflower mosaic virus 35S promoter followed by the 5'-untranslated sequence of tobacco mosaic virus RNA. The chimeric gene was introduced into Nicotiana benthamiana by Agrobacterium-mediated gene transfer, and the expression of CP gene was verified by Western blot analysis. Self-fertilized R1 progeny expressing detectable levels of CP showed significant delay in symptom development, as well as an attenuation of the symptoms.

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Key words: bean yellow mosaic virus, nuclear inclusion protein, coat protein, nucleotide sequence, transgenic plant, coat protein-mediated resistance.

INTRODUCTION

The potyviruses are the largest and economically the most important group of plant viruses[15]. The genome of potyvirus is composed of a single positive-sense RNA of approximate 10 kb with a genome-linked protein (VPg) at the 5'-terminus and a poly(A) tract at the 3'-terminus[7]. The complete nucleotide sequences of several potyviruses, tobacco etch virus (TEV)[1], tobacco vein mottling virus (TVMV)[6], plum pox virus (PPV)[22], potato virus Y (PVY)[23], pea seed-borne mosaic virus (PsbMV)[43], pepper mottle virus (PepMoV)[43], papaya ringspot virus (PRSV)[46] and turnip mosaic virus (TuMV)[29], have been determined.

Bean yellow mosaic virus (BYMV), an important member of the potyvirus group, infects leguminous crops and several ornamental plants and is easily transmitted by over 20 species of aphids in a non-persistent manner[8]. Recently, a necrotic strain of BYMV has spread and caused extensive damage to broad bean in Tohoku district[12], especially in Miyagi Prefecture. The general presence of inoculum...
around broad bean fields, which is thought to result from the frequent infection of clover by BYMV, makes it difficult to control broad bean mosaic and necrotic disease caused by the virus.

Recently, it was demonstrated that transgenic plants expressing viral coat protein (CP) genes are resistant to infection by the homologous virus. This type of pathogen-mediated resistance is known as CP-mediated resistance and has been demonstrated for several virus groups. Moreover, the transgenic plants expressing a potyvirus CP gene were shown to be resistant to infection against other heterologous potyviruses. Taking advantage of this wide spectrum of resistance, it would be possible, by introduction of a single potyvirus CP gene, to develop broad bean that is resistant against those heterologous potyviruses known to infect wild type, namely PSbMV, BYMV and clover yellow vein virus (ClYVV).

As a first step toward determining whether BYMV CP could be used to produce leguminous crops resistant to infection against potyviruses, we cloned and sequenced the 3′-terminal 4,306 nucleotides of BYMV, which include the nuclear inclusion protein (NiA, NiB) and CP genes, and introduced the CP gene into *Nicotiana benthamiana*, which is susceptible to infection by several different kinds of potyviruses. We report here the nucleotide sequence of 3′-terminal 4,306 nucleotides, and the transfer and expression of BYMV CP gene in the transgenic plants. Furthermore, we demonstrate that some of these transgenic plants are resistant against homologous BYMV.

**MATERIALS AND METHODS**

**cDNA cloning.** MB4 strain of BYMV was propagated in broad bean (*Vicia faba*). Viral RNA was isolated from freshly prepared virus described by Robaglia et al. The cDNA cloning was based on the method of Gubler and Hoffman using oligo(dT) primers and Amersham supplies. The cDNA was given poly(C) tails, ligated into dG-tailed, PstI-cut pUC9 and used to transform competent JM109 *Escherichia coli* cells. The resulting library was screened for length of the inserted cDNA by digesting mini-preparations of plasmid DNA with PstI.

**Sequencing.** The clone pMB6 contained the largest insert (4.3 kb), and was chosen for nucleotide sequence analysis. After subcloning the insert into pBluescript SK+, nested deletions were made in both directions according to the Stratagene protocol based on Yanish-Perron et al. Both strands of the cDNA were sequenced by the dideoxynucleotide chain-termination methods using single-strand DNA generated by superinfection of transformed cells with the helper phage VCSM3. Nucleotide sequences were analyzed using the DNASIS sequence analysis program (Hitachi). The generated amino acid sequences of NiA and NiB protein regions were compared to other potyviruses, PVY, PPV, TEV and TVMV, and that of CP was compared to other BYMV strains, GDD, Danish, CS and S.

**Construction of transformation vector.** Two oligomers, MBCP5 (5′-CCCGGATCCACCATG-3′) and MBCP3 (5′-CCCGAGCTCCTAAATACGAAC-3′), were used for amplification of CP-coding region and for introduction of the translation initiation codon to the 5′-end and for providing restriction sites to both ends. Polymerase chain reaction (PCR) was performed as described by Saiki et al. The mutagenized CP gene was inserted into BamHI/SacI site of binary vector pBE211. The resultant plasmid, designated pBE211-MBCP, was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by electroporation.

**Plant transformation.** Leaf disk transformation of *N. benthamiana* by *A. tumefaciens* was done as described previously. Kanamycin-resistant plantlets were grown in the growth room and the integration of CP gene into chromosome DNA was checked by PCR amplification of CP-coding region using the above two primers.

**Western blot analysis.** Leaf samples from transgenic plants were ground in Laemmli loading buffer (1 : 4 w/v) and immediately boiled for 5 min. The extracts were centrifuged and 5 μl of supernatant was subjected to electrophoresis in 12.5% polyacrylamide gel. Proteins were electrophoretically transferred onto nitrocellulose membrane as described by Towbin et al. BYMV CP was detected with anti-BYMV-MB4 polyclonal antibodies followed by alkaline phosphatase-conjugated goat.
anti-rabbit antibodies.

**Infection of transgenic plants with BYMV.** Transgenic plants expressing BYMV CP, as determined by Western blot analysis, were self-pollinated. Kanamycin-resistant R1 seedlings with 4–5 leaves were dusted with carborundum on the youngest expanded leaves and rubbed with 1 : 5 dilution of inocula from BYMV-MB4 infected broad bean leaf extract in 10 mM phosphate buffer, pH 7.0. Symptom development was recorded from 5 to 17 days after inoculation.

**RESULTS**

**Sequencing of the 4,306 nucleotides coding Nla, Nlb and CP of BYMV-MB4 RNA**

The nucleotide sequence of cDNA clone pMB625 is shown in Fig. 1 along with its predicted amino acid sequence. It was found to contain an open reading frame 4,131 nucleotides long, with the capacity to encode 1,377 amino acids. Based on comparisons with other potyvirus genome organizations, it was expected to contain 6 K, Nla, Nlb and coat protein regions. Recently, the complete nucleotide sequences of several potyviruses have been determined and Nla-proteinase cleavage sites of the polyproteins determined or proposed(Takami et al., 1992; Takami et al., 1993). Based on these informations and on sequence homologies of the polyproteins, four Nla-proteinase cleavage sites are identified in the BYMV-MB4 1,377 amino acids polyprotein (Table 1). The sequence F(Q or E)/(S or G) is proposed to be a consensus cleavage sequence for the Nla-proteinase of BYMV. However, valine found in position P4 in seven potyviruses was not found in BYMV. Recently, the N-terminal domain of Nla protein has been reported to be a viral VPg²,³. The internal cleavage site of these two Nla protein domains was also found in Nla protein of BYMV at positions 342–343 (Table 1). Thus, BYMV Nla can also be divided into two domains comprising two distinct proteins, VPg and proteinase.

The proposed Nla protein of BYMV showed 47, 51, 50 and 51% amino acid sequence identity with the corresponding proteins of PVY, PPV, TEV and TVMV, respectively. The catalytic triad (H, D, C) of Nla-proteinase⁸ was also present in BYMV (polyprotein positions 388, 423 and 493 : Fig. 1).

The most conserved potyviral protein is Nlb, a putative polymerase. The BYMV Nlb showed a high degree of amino acid sequence similarity with the corresponding proteins of four other potyviruses, ranging from 62% with TEV and TVMV to 65% with PVY and PPV. The consensus motifs (YCDADGS- QFDSSL, GNNSGPSTVVDNTLMV, NGDDL and K), responsible for the RNA-dependent RNA polymerase function¹⁷ were also present in BYMV (polyprotein position 830-842, 893-909, 935-939 and 974 : Fig. 1).

BYMV-MB4 CP showed 54, 56, 60 and 65% amino acid similarity with the CP of TVMV, PPV, PVY and TEV, respectively. The CPs of four other isolates of BYMV have been sequenced to date: American GDD¹⁴, Danish⁶, Japanese CS³⁸ and Australian S⁴⁰. The amino acid sequences of CPs from these five strains were identical in length (273 aa), and MB4 CP showed 93, 95, 88 and 92% identity with those of GDD, Danish, CS and S, respectively. The major difference between the five isolates was found in the N-terminus, whereas the center and C-terminus were highly conserved, as previously described for other potyviruses. The amino acid sequence DAG in the N-terminal region of CP, which is present in

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The table above shows the proposed cleavage sites recognized by the Nla proteinase in the BYMV-MB4 polyprotein.
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Fig. 1. The nucleotide and predicted amino acid sequences of the 3'-terminal 4,306 nucleotides of BYMV-MB4. Potential proteolytic cleavage sites are indicated by arrowheads.
Fig. 2. Modification of the 5’-terminal sequence of the BYMV CP gene and diagrammatic representation of plant transformation vector pBE2113-MBCP. E35S: enhancer sequence of CaMV 35S promoter; P35S: CaMV 35S core promoter; \( \Gamma \): TMV 5’-untranslated sequence; NOS3’: NOS terminator.

Fig. 3. Western blot analysis of transgenic N. benthamiana plants. Lane 1: Non-transformed plant; lane 2-5: CP-transformed R1 progeny (B801: lanes 2 and 3; B805: lanes 4 and 5); Lane 6: Purified BYMV-MB4 (4 ng CP). In lane 1-5, SDS-soluble proteins from 1 mg leaves (fresh weight) were applied.

all potyviruses except for the BYMV subgroup and is associated with aphid transmission, was not found in BYMV-MB4, and instead NAG was found at the comparable position (polyprotein position 1,111-1,113, Fig. 1).42)

The stop codon at position 4,133-4,135 was followed by 3’-untranslated region (3’UTR) of 171 bases, excluding the poly(A) tail. Surprisingly, the 171 bases of 3’UTR of MB4 isolate were completely identical with those of the Danish isolate.

Expression of BYMV CP gene in transgenic N. benthamiana

Because the potyvirus CP is encoded as part of a polyprotein, its message does not contain a translation initiation codon. Therefore, the coat protein coding region was mutagenized in vitro using PCR to introduce the ATG codon to the 5’ end (Fig. 2). The mutagenized CP gene was cloned into pT7/T3-19 (BRL) to produce the CP messenger RNA driven by T7 promoter in vitro. The in vitro transcribed CP mRNA was checked for production of the appropriate CPs in rabbit reticulocyte lysate system in vitro (data not shown).

The engineered BYMV CP gene was removed from pT7/T3-19MBCP by digesting with BamHI/SacI and cloned into a high-expression binary vector, pBE2113. The resulting plasmid, pBE2113-MBCP, was used to transform N. benthamiana mediated by A. tumefaciens. Eleven independently regenerated transformants of N. benthamiana were obtained. The BYMV CP gene was integrated into the genomic DNA in eight of these transformants, and three produced detectable levels of BYMV CP as observed by Western blot analysis. Transformant B801 and B805, used for protection experiment, expressed the appropriate size CP (31kDa) which was the same as that of purified BYMV-MB4, and both levels of accumulated CP were roughly equal (data not shown). Furthermore, R1 progeny of B801 and B805 also produced equal amounts of CP relative to their parental plants (Fig. 3).

Protection of R1 progeny against homologous BYMV

Kanamycin-resistant R1 seedlings, plant line B801 and B805, were inoculated with BYMV-MB4 at 1:5 dilution of infected broad bean leaf extract. Severe systemic mosaic symptoms developed in all of the control (non-transformed) plants within 11 days after inoculation, whereas the progeny of both B801 and B805 showed significant levels of resistance (Fig. 4). The progeny of B805 was more resistant than
that of B801. However, in two of the five B801 plants that showed symptoms 11 days after inoculation, mild mosaic symptoms faded as the leaf aged and the symptoms of other three plants were attenuated by comparison with control plants.

DISCUSSION

In this report, we described the cloning and sequencing of 3' terminal 4,306 nucleotides of BYMV-MB4 and the expression of its CP gene in transgenic plants. The region of the genome included NIa (VPg and proteinase), NIb (putative polymerase) and CP. We employed the CP gene to produce transgenic plants resistant to virus infection. The other two cistrons, however, are useful: NIa can serve as a specific proteinase for protein engineering, and transgenic plants expressing NIa protein were shown to be resistant to homologous virus. Furthermore, in PVX, it was shown that transgenic plants expressing a modified viral replicase were highly resistant to infection by PVX.

NIa proteinase of potyvirus has been shown to cleave at six locations along the polyprotein, P3/CI, CI/6K, 6K/NIa-VPg, NIa-VPg/NIa-Pro, NIa-Pro/NIb and NIb/CP. We have found five potential cleavage sites in BYMV-MB4 polyprotein, excluding P3/CI site (Table 1). Each of the proposed BYMV-MB4 NIa cleavage sites occurred between (Q or E) and (S or G), as demonstrated for other potyviruses, whereas phenylalanine (F) at the position P2 was specific for BYMV-MB4. This consensus sequence F (Q or E)/(S or G) in BYMV-MB4 is suitable for the cleavage sequences between NIb and CP not only in other strains of BYMV but in three strains of CIYVV.

The CP of BYMV-MB4 showed 88-95% amino acid similarity with four BYMV strains, as previously reported. Most notably, the CPs of MB4 and Danish demonstrated 95% amino acid homology, and showed complete identity for their respective 3'UTR nucleotide sequences. Since the homology of potyviral 3'UTR has been shown to reflect their genetic relatedness, the two isolates, Japanese MB4 and Danish, can be considered genetically closely related.

Using Agrobacterium-mediated transformation, we obtained transgenic N. benthamiana expressing the BYMV CP gene. The plants that accumulated detectable levels of CP showed significant levels of resistance against homologous virus. In our experiment, B805 showed a higher level of protection than B801, although both levels of accumulated CP made little difference (Fig. 3). In the case of TMV, the degree of protection was directly proportional to the levels of accumulated CP, whereas no correlations in the levels of accumulated CP with the degree of protection were observed in some viruses: such as SMV, PVY, PPV and CMV. No consensus has yet been obtained to correlate the levels of accumulated CP with the degree of protection, so there would be some risks associated with screening for resistant plants based on the expression levels of the CP gene without protection tests.

We intend to produce a transgenic broad bean resistant to potyviral infections. In Tohoku district, BYMV and CIYVV are economically the most important viruses in broad bean, especially the latter since it causes severe necrosis and thus serious damage to broad bean. CIYVV belongs to the BYMV.
subgroup and its CP shows 70–77% amino acid homology to the protein from four strain of BYMV, so it is needed to examine whether the transgenic plants expressing BYMV CP could be protected from CIYVV infection.

**Literature cited**


和文摘要

中村茂雄・本崩良三・宇垣正志・大島正弘・大橋拓子：インゲンマメ黄斑モザイクウイルス (BYMV) ゲノム RNA 3'末端の塩基配列と外被タンパク質遺伝子導入 Nicotiana benthamiana の作出

BYMV のゲノム RNA 3'末端から 4,306 塩基の塩基配列を決定した。第 2 塩基から第 4,132 塩基まで一本のオープン・リーディング・フレームが見いだされた。既報の potyvirus のゲノム構造と N1a プロテアーゼによる認識配列を参考にして、本ウイルスのコードする N1a プロテアーゼ切断部位を予想し、ゲノム構造を推察したところ、この領域には CI の一部 (98aa), 6K (53aa), N1a (434aa), N1b (519aa) および外被タンパク質 (CP, 273aa) がコードされていると考えられた。CP は既に塩基配列が報告されている他の BYMV 4 系統とアミノ酸レベルで 88-95% のホモロジーを示した。更に、本ウイルスに対する抵抗性植物作出のため、本 CP 遺伝子に翻訳開始コドンを導入し、アグロバクテリアム感染法によって Nicotiana benthamiana を形質転換した。得られた形質転換植物について、核ゲノムへの CP 遺伝子の組み込みと、タンパク質レベルでの発現を確認した。また自殖次世代においても安定して CP を発現し、BYMV 感染に対して抵抗性を有していることが明らかとなった。