Systemic production of foreign peptides on the particle surface of tobacco mosaic virus

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Abstract By using a new tobacco mosaic virus (TMV) vector [Hamamoto, H., et al. (1993) Bio/Technology 11, 930–932], we have constructed TMV particles which present three different kinds of epitopes, two of them from influenza virus hemagglutinin (HA), and one from human immunodeficiency virus type I (HIV-I) envelope protein, on the surface of the particles. Each of these TMV particles reacted with each anti-peptide antiserum. These results suggest that this TMV vector can be used as an antigen presentation system.

Key words: Foreign peptide; Fused protein; Virus particle; Tobacco mosaic virus

1. Introduction

An attractive approach for an effective peptide vaccine is presenting an epitope on the surface of a particle structure, and considerable interest has been focused on the biochemical linking of the epitope to a carrier protein which is able to form a particle structure. The use of virus coat proteins of bacteria [1–3], animals [4–7] and plants [8–10] as carrier proteins has been reported. Recently, it has been shown that cowpea mosaic virus (CPMV) in which epitopes from animal viruses were inserted into the small coat protein, can present the epitopes on the surface of the virus particle, and the inserted epitopes are immunogenic in rabbits [10].

We have previously developed a tobacco mosaic virus (TMV) vector which can produce both intact coat protein (CP) and fused protein (FP) consisting of CP and angiotensin I-converting enzyme inhibitor peptide (ACEI) by inserting a translational read-through signal of a 'leaky' UAG (amber) stop codon downstream of the CP gene [11]. This vector could form virus particles and spread systemically in tobacco and tomato plants. Moreover, protein analysis of progeny virus particles showed not only the presence of intact CP, but also that of FP, CP–ACEI [11]. Because ACEI was fused to the C-terminus of the CP, and the C-terminus portion of the CP protrudes outwards from the TMV particle [12,13], it is suspected that ACEI is located on the surface of the virus particle.

In this paper, we describe the construction of TMV particles which present epitopes from influenza and human immunodeficiency viruses on the surface of the particles. The peptide sequences used for the particles consisted of residues 91–108 (pep18), 139–146 (pep8) from influenza virus HA [14], and the principal neutralization determinant consisting of 13 residues (pep13) from HIV-I gp120 [15]. It has been shown that antibodies against these peptides had binding capacity to the virus [14,15].

2. Materials and methods

2.1. Plasmid construction, in vitro transcription, inoculation of plants, and virus particle purification

The construction of pTLW3 and pTLRTB has been described previously [11]. Pairs of partially complementary oligonucleotides: dGACCTCTGCACCTGCATCATAGCAATTATCTAAAGCTTTCTCTAATTGTTACCCTTACGATGTTCCTGATTACGCATCTTTATAAATGCA and dTTTATAAAGATGCGTAATCAGGAACATCGTAAGGGTAACAATTAGAGAAAGCTTTAGATAATTGCTATGATGCAGGTGCAGAG for pTLHA1, dGACCTCTGCACCTGCATCATAGCAATTATGTAAAAGAGGTCCTGATTCTGGTTAAATGCA and dTTTAACCAGAATCAGGACCTCTTTTACATAATTGCTATGATGCAGGTGCAGAG for pTLHA2, dGACCTCTGCACCTGCATCATAGCAATTAAAATCTAGAATACAAAGAGGTCCTGGTAGAGCATTTGTTTAAATGCA and dTTTAAACAAATGCTCTACCAGGACCTCTTTGTATTCTAGATTTTAATTGCTATGATGCAGGTGCAGAG for pTLHIV were synthesized, respectively, to construct vectors. These oligonucleotides replaced the *Ava*II (position 6160)–*Nsi*I (position 6183) fragment of pTLW3 in the same manner as pTLRT, as described previously [11]. In vitro transcription, inoculation of plants, and virus particle purification, were carried out as described previously [11].

2.2. Western blot analysis

Samples of 1 μg of virus particle were separated by 12.5% SDS-PAGE and the proteins were transferred to PVDF membranes. A ProtoBlot Western Blot AP system (Promega) was used for immunodetection. Anti-ACEI, anti-pep18, anti-pep8 and anti-pep13 antisera were raised in rabbit against synthetic peptide–keyhole limpet hemocyanin (KLH) conjugate.

2.3. Trypsin treatment

25 μg of purified virus particles or purified free CP were treated with 0.5 μg of trypsin or without trypsin in 50 mM Tris-HCl (pH 7.5) buffer at 30°C for 5 min. After treatment with trypsin, 1 μg of the virus particles was used for Western blot analysis.

2.4. Precipitin reaction

A layer of antiserum was covered with a layer of virus particle solution at concentrations of 4 mg/ml in 0.2 ml polypropylene tube. Photographs were taken after incubation at room temperature for 15 min.

3. Results

3.1. Vector construction

TMV RNA vectors were transcribed from their template plasmids in vitro [11]. pTLW3 [11] is the standard template

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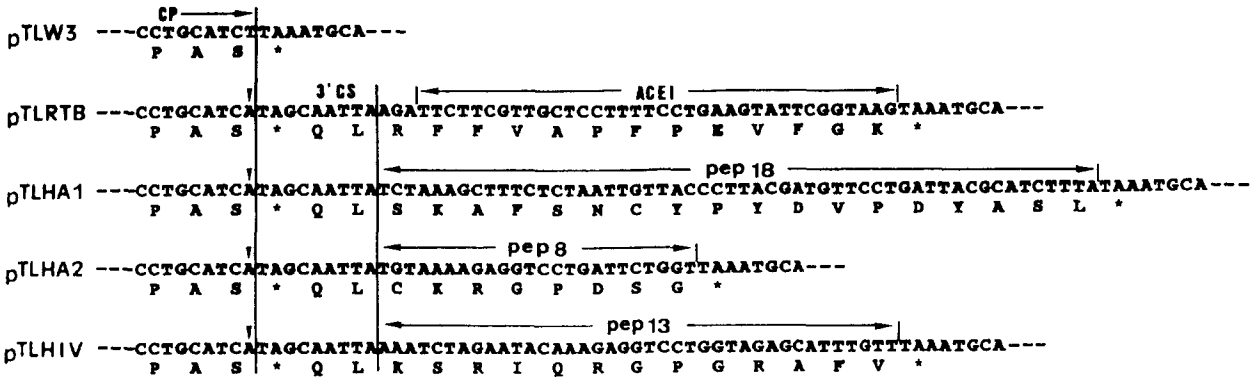

Fig. 1. The sequences around the stop codon for CP genes of template plasmids (pTLW3, pTLRTB, pTLHA1, pTLHA2 and pTLHIV). Amino acids are shown under nucleotide sequences by one-letter symbols. In pTLRTB, pTLHA1, pTLHA2 and pTLHIV, the 3′ terminal T of the CP gene (pTLW3) was changed to A, indicated by filled triangles, to increase the read-through efficiency [11]. In pTLRTB, a codon for Arg, AGA, was placed between 3′ context sequence (3′CS) and the ACEI gene to enable the release of ACEI by treatment with trypsin. The 3′CS allows read-through of a 'leaky' UAG (amber) stop codon [16]. Asterisks indicate the stop codons.

plasmid for wild-type TMV RNA. pTLRTB [11] is the template plasmid used to produce both intact CP and CP–ACEI. pTLHA1 was designed for production of both intact CP and CP–pep18. pTLHA2 was designed for both intact CP and CP–pep8, and pTLHIV for both intact CP and CP–pep13 (Fig. 1). In pTLHA1, pTLHA2 and pTLHIV, oligonucleotides encoding the peptides were inserted just after the 3′ context sequence which allows read-through of a 'leaky' UAG (amber) stop codon [16]. These RNA transcripts will be referred to by deleting the prefix 'p' from their template designations.

3.2. Systemic infectivity of TMV vectors in tobacco plants

To investigate the ability of TLHA1, TLHA2 and TLHIV vectors to spread systemically in tobacco plants, 20 ng of TMV RNA vectors were encapsidated with CP in vitro and inoculated onto leaves of five tobacco (*Nicotiana tabacum* cv. Samsun) plants as described previously [11]. Inoculation of these vectors resulted in mosaic symptoms 2 or 3 weeks after the inoculation in the upper non-inoculated leaves. By Western blot analysis, both intact CP and fused protein were detected in the inoculated and upper non-inoculated leaves of TLHA1-, TLHA2- and TLHIV-infected plants (data not shown), as in the case of TLRTB-infected plants [11]. This result indicates that TLHA1, TLHA2 and TLHIV vectors can form virus particles and spread systemically in plants, as in the case of TLRTB vector.

3.3. Purification of virus particles

One month after the inoculation, virus particles were purified from upper non-inoculated leaves as described previously [11]. From 10 g of tobacco leaves, 18 mg of TLW3, 12 mg of TLRTB, 8 mg of TLHA1, 20 mg of TLHA2 and 16 mg of TLHIV virus particles were obtained. Proteins in these virus particles were examined by Western blot analysis using an anti-TMV antiserum. All virus particles, except the TLW3 particles, included not only intact CP but also FP (Fig. 2A). To confirm

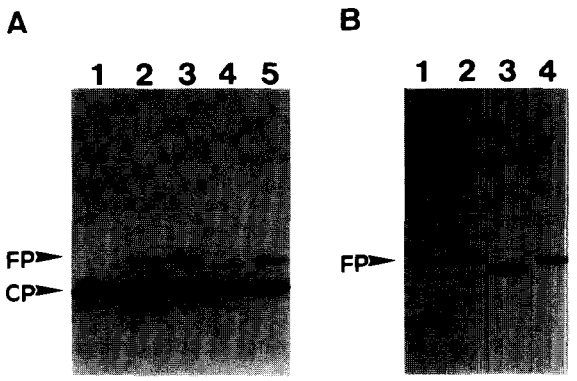

Fig. 2. Western blot analysis of the proteins of virus particles using anti-TMV antiserum (A), anti-ACEI (B, lane 1), anti-pep18 (B, lane 2), anti-pep8 (B, lane 3) and anti-pep13 (B, lane 4) antiserum. Samples are from the virus particles of TLW3 (A, lane 1), TLRTB (A, lane 2; B, lane 1), TLHA1 (A, lane 3; B, lane 2), TLHA2 (A, lane 4; B, lane 3) and TLHIV (A, lane 5; B, lane 4). The positions of CP and FP are shown on the left.

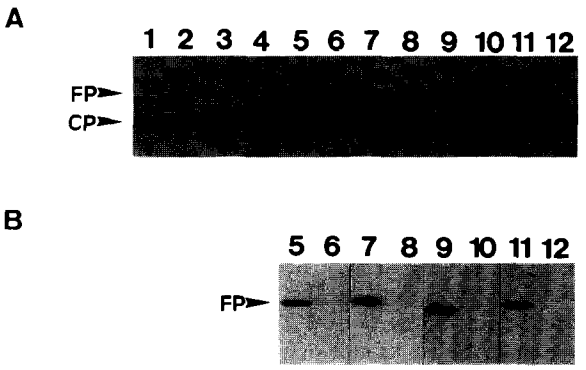

Fig. 3. Western blot analysis of the proteins of virus particles treated with trypsin using anti-TMV antiserum (A), anti-ACEI (B, lanes 5 and 6), anti-pep18 (B, lanes 7 and 8), anti-pep8 (B, lanes 9 and 10) and anti-pep13 (B, lanes 11 and 12) antiserum. Samples are from purified free CP (lanes 1 and 2), and the virus particles of TLW3 (lanes 3 and 4), TLRTB (lanes 5 and 6), TLHA1 (lanes 7 and 8), TLHA2 (lanes 9 and 10) and TLHIV (lanes 11 and 12). The samples were treated with trypsin (lanes 2, 4, 6, 8, 10 and 12), or without trypsin (lanes 1, 3, 5, 7, 9 and 11). The positions of CP and FP are shown on the left.

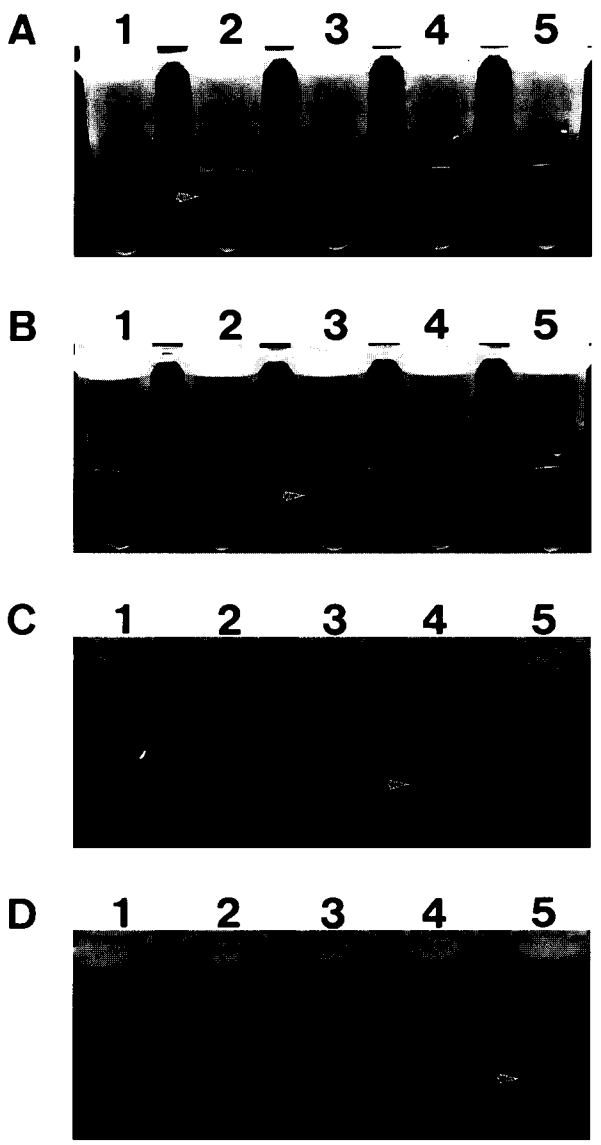

Fig. 4. Precipitin reaction (ring test) using anti-ACEI (A), anti-pep18 (B), anti-pep8 (C) and anti-pep13 (D) antiserum. A layer of antiserum was covered with a layer of TLW3 (tube 1), TLRTB (tube 2), TLHA1 (tube 3), TLHA2 (tube 4) and TLHIV (tube 5) virus particle solution. The positions of the precipitin ring are indicated by arrowheads.

that FPs were CP–ACEI, CP–pep18, CP–pep8 and CP–pep13, respectively, antiserum against ACEI, pep18, pep8 and pep13 were used to perform Western blot characterization of these proteins. Anti-ACEI, anti-pep18, anti-pep8, and anti-pep13 antiserum specifically bound to FPs in TLRTB, TLHA1, TLHA2 and TLHIV virus particles, respectively (Fig. 2B). Further purification through sucrose density gradient centrifugation [17] did not prevent the FPs from appearing in the virus particles (data not shown). Thus FPs are incorporated into the particles, and are not just adhering to their surface. The relative amount of FP-to-CP included in virus particles was about 1:20 and the same as in leaf extracts. These results indicate that FPs are incorporated into the virus particles as efficiently as CP.

3.4. Properties of virus particles

To investigate whether the foreign paptides of FPs in virus particles locate on the surface of the particles, the particles were treated with trypsin. An Arg residue was placed just N-terminus of the ACEI sequence in TLRTB, and the peptides derived from HA and gp120 in TLHA1, TLHA2 and TLHIV contained Arg or Lys residues. Since TMV particle is known to be resistant to treatment with trypsin [18], it is likely that only the sequences locating on the surface of the virus particles are digested with trypsin. Proteins of virus particles treated with trypsin were examined by Western blot analysis using an anti-TMV antiserum. The intact CP in the virus particles was not digested with trypsin (Fig. 3A, lanes 4, 6, 8, 10 and 12), and purified free CP was completely digested with trypsin (Fig. 3A, lane 2). On the other hand, FPs in the virus particles were susceptible to trypsin, resulting in shift of their mobility between the original FPs and intact CP (Fig. 3A, lanes 6, 8, 10 and 12). Anti-ACEI, anti-pep18, anti-pep8 and anti-pep13 antiserum did not bind to the digested FPs (Fig. 3B, lanes 6, 8, 10 and 12).

Precipitin reactions were observed specifically between anti-ACEI antiserum and TLRTB virus particle, anti-pep18 antiserum and TLHA1 virus particle, anti-pep8 antiserum and TLHA2 virus particle, and anti-pep13 antiserum and TLHIV virus particle, respectively (Fig. 4). These results support the notion that foreign peptides of FPs in virus particles are located on the surface of the particles, as depicted in Fig. 5.

4. Discussion

TMV vectors we have developed can form virus particles and the virus particles include both intact CP and FP (Fig. 2). The yield of the virus particles is about the same as that of wild-type TMV, and propagation and purification of the particles are very easy. In addition, no loss of the foreign genes inserted in the vectors was observed after three serial passages. Thus this

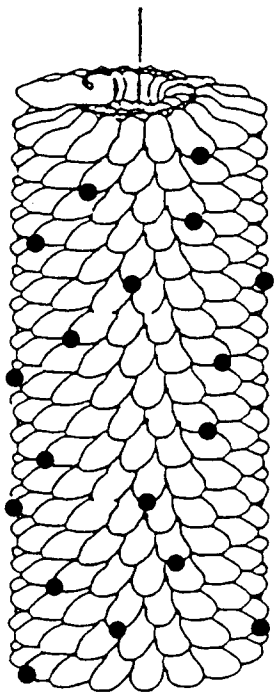

Fig. 5. A model for the virus particle carrying foreign peptides on the surface. The foreign peptides of the FPs are indicated by filled circles.

type of TMV vector grows stably, and large amounts of virus particles can be obtained, indicating that foreign peptides can be as easily purified as virus particles from the inoculated plants.

The most attractive feature of the virus particles is that foreign peptides as long as 21 amino acids can be presented on the particle surface (Figs. 3 and 4). We think that this feature makes it possible to present an epitope to the immune system and this TMV vector system may be a tool for the production of vaccines in plants.

We are working on increasing read-through efficiency and on determining how large a peptide can be presented on the surface of the virus particles.

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