Production of enkephalin in tobacco protoplasts using tobacco mosaic virus RNA vector

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To examine the validity of the strategy to express a foreign gene as a fusion protein with the coat protein (CP) of tobacco mosaic virus (TMV), we have constructed ENK RNA by using an in vitro transcription system of TMV RNA. ENK RNA differs from TMV RNA only in that ENK RNA carries an additional sequence coding for Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) (Enk) with a preceding in-frame methionine just before the termination codon of CP gene. In protoplasts inoculated with ENK RNA, CP + Enk fusion protein accumulated as the major protein.

Enkephalin; Fusion protein; Transcription system, in vitro; RNA vector; Tobacco mosaic virus

1. INTRODUCTION

In vitro transcription of RNA viruses by which infectious transcripts can be generated from cDNA clones is a powerful tool to study viral replication and has been developed for many positive strand RNA viruses, including bromo mosaic virus [1], tobacco mosaic virus (TMV) [2,3], cowpea chlorotic mottle virus [4], turnip yellow mosaic virus [5], poliovirus [6] and Sindbis virus [7]. This system also facilitates the use of viral RNAs as expression vector [8–10]. TMV is a plant virus with a messenger-sense, single-stranded RNA of about 6400 nucleotides [11]. Previously, we constructed chimeric TMV RNAs carrying the chloramphenicol acetyltransferase (CAT) gene instead of the coat protein (CP) gene by using an in vitro transcription system [3] and reported expression of CAT gene in tobacco plants [9]. While CP accumulates to several milligrams per gram of TMV-infected tobacco leaf tissue [12], only about 1 μg of CAT per gram of inoculated leaf tissue was produced [9]. As CP is produced in large quantity and is stable in TMV-infected tobacco plants, we examined the possibility to express a foreign gene as a fusion protein with CP. In this paper, we report expression of Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) (Enk), a pentapeptide with opiate-like activity [13], as a fusion protein with CP in tobacco protoplasts by means of a TMV RNA vector.

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2. MATERIALS AND METHODS

2.1. *Plasmid construction*

All plasmids were constructed from pLFW3 [3]. The TMV sequence in pLFW3 is numbered from the 5′ G residue [14].

pLCLE was constructed from pLFW3 by replacing the *Ava*II-(residue 6160)/*Nsi*I(6183) fragment of pLFW3 with two partially complementary synthetic oligonucleotides, dTTTAGAGGAAGCCACCGTACATAGATGCAGGTGCAGAG and dGACCTCTGCACCTGCATCTATGTACGGTGGCTTCCTCTAAATGCA. pLDCGA3 was constructed in the same manner as pLCD147 as previously described [15].

2.2. *Transcription, reconstitution and inoculation*

pLFW3 and its derivatives were linearized at the unique *Mlu*I site downstream of TMV cDNA and used to prepare in vitro transcripts [3]. In vitro transcripts were reconstituted with CP of TMV-OM (a common strain) in vitro and inoculated onto tobacco leaves according to Meshi et al. [3]. *N. tabacum* L. cvs. Xanthi nc and Samsun were used as a local lesion host and a systemic host, respectively. Progeny virus were extracted from the inoculated leaves or the upper, uninoculated leaves of *N. tabacum* L. cv. Samsun [16]. For back-inoculation, excised local lesions were homogenized with 10 mM phosphate buffer (pH 7.0) and the homogenate was used as inoculum.

2.3. *Analysis of protein synthesis in protoplasts*

Protoplasts were isolated from suspension-cultured cells of *N. tabacum* L. cv. BY-2 [17]. In vitro transcripts were inoculated into tobacco protoplasts by electroporation as described by Watanabe et al. [17] except that DNase I treatment after in vitro transcription was omitted. For analysis of TMV-specific proteins, protoplasts were labeled by adding [^{35}S]-methionine to the culture medium and the lysates of the inoculated protoplasts were subjected to NaDodSO$_4$/12% polyacrylamide gel electrophoresis, followed by staining with Coomassie blue or fluorography [18].

2.4. *Protein isolation and amino acid sequence determination*

10^6 protoplasts were inoculated with about 2 μg of in vitro transcript derived from pLCLE, collected at 24 h post-inoculation and subjected to NaDodSO$_4$/12% polyacrylamide gel electro-

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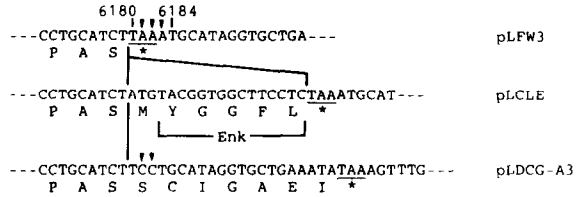

Fig. 1. The sequences around the termination codons of CP or its derivative genes of pLFW3, pLCLE and pLDCG-A3. The TMV sequence of 6384 nucleotides is numbered from the 5′ G residue [14]. The termination codons of CP or its derivative genes are underlined. Amino acids are shown under the nucleotide sequences by one-letter symbols. In pLDCG-A3, the AAA sequence (residues 6181–6183) of pLFW3, indicated by arrowheads, was substituted with the CC sequence indicated by arrowheads.

phoresis. After staining with Coomassie blue, the gel band containing CP + Enk fusion protein was excised and the protein was eluted with H_2O. CP + Enk fusion protein was cleaved by cyanogen bromide (BrCN) in 70% (v/v) formic acid and dried in vacuo. The BrCN-treated CP + Enk was dissolved with 0.1% (v/v) trifluoroacetic acid (TFA), and Enk fragment was separated on a μBondapak C_{18} column by reverse phase HPLC with a gradient of acetonitrile in 0.1% (v/v) TFA. The authentic biologically active Enk (Peptide Institute Inc.) was used to obtain the retention time reference on this column under the same conditions. The amino acid sequence was determined by using an Applied Biosystems Inc. 470A gas phase sequencer.

3. RESULTS

3.1. *Plasmid construction*

Considering that the C-terminal portion of CP projects outwards from the virus particle [19], it is likely that CP derivatives which retain an additional short peptide sequence at the C-terminus could still form virus particles. In this case, the CP fusion proteins could be easily recovered by purifying virus particles and, since CP is produced in large quantity and is stable in TMV-infected tobacco plants [12], large amounts of the fusion proteins might be obtained. To test this possibility, we have constructed two TMV RNA derivatives in which the altered CP gene codes for an additional amino acid sequence of 6 or 7 residues after the authentic C-terminus.

pLFW3 is a plasmid carrying a full-length cDNA copy of TMV-L (a tomato strain) RNA immediately downstream of the modified P_R promoter of lambda DNA [20] and its *MluI* digest is used as the standard in vitro transcription template of infectious TMV-L RNA [3]. pLCLE differs from pLFW3 only in that pLCLE carries an additional sequence coding for Enk with a preceding in-frame methionine just before the termination codon of CP gene (Fig. 1). CP + Enk fusion protein is expected to be produced in the cells infected with in vitro transcript derived from pLCLE. A methionine was placed between CP and Enk sequences in order to isolate Enk by BrCN cleavage. In pLDCG-A3, the AAA sequence (residues 6181–6183) near the termination codon of CP gene of pLFW3 was substituted by the sequence CC and thus the in vitro transcript derived from pLDCG-A3 is expected to produce a CP derivative with an extra amino acid sequence of Ser-Cys-Ile-Gly-Ala-Glu-Ile at its C-terminus (Fig. 1). In vitro transcripts derived from pLFW3, pLCLE and pLDCG-A3 are designated W3, ENK and CG RNA, respectively.

3.2. *Responses of tobacco plants*

To test infectivity of ENK and CG RNAs, in vitro transcripts were inoculated onto *N. tabacum* L. cv. Xanthi nc after reconstitution with CP. Both ENK and CG RNAs produced local lesions similar to those of W3 RNA on the inoculated leaves (Fig. 2). When inoculated onto *N. tabacum* L. cv. Samsun, CG RNA gave rise to typical systemic mosaic symptoms on the upper, uninoculated leaves about one week post-inoculation as W3 RNA (data not shown). From the systemic plants inoculated with CG RNA, virus particles were recovered from both the inoculated and the upper, uninoculated leaves, and the constituent protein migrated slightly more slowly than CP produced by W3

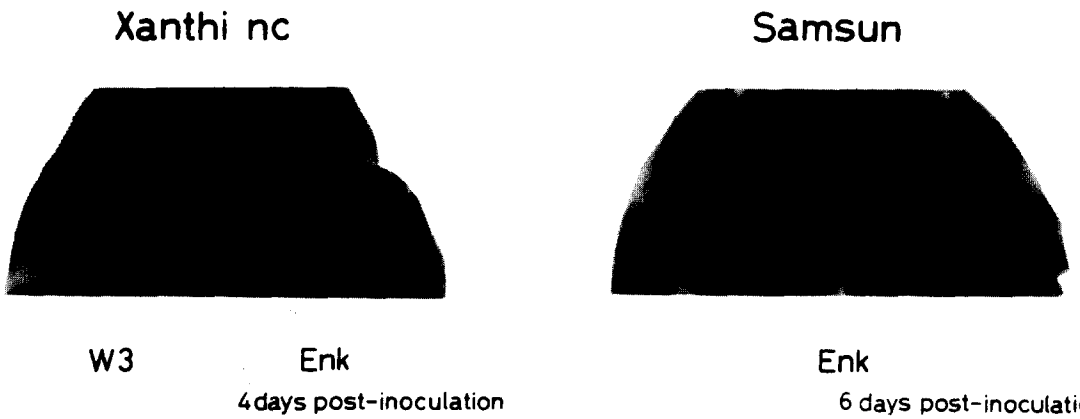

Fig. 2. Local lesions on the inoculated leaves of *N. tabacum* L. cvs. Xanthi nc and Samsun. W3 or ENK RNA was reconstituted with CP in vitro and then used as inocula [3]. On the Xanthi nc leaf, the left half was inoculated with W3 RNA and the right half with ENK RNA. The Samsun leaf was inoculated with only ENK RNA. Photographs were taken at 4 or 6 days post-inoculation, respectively.

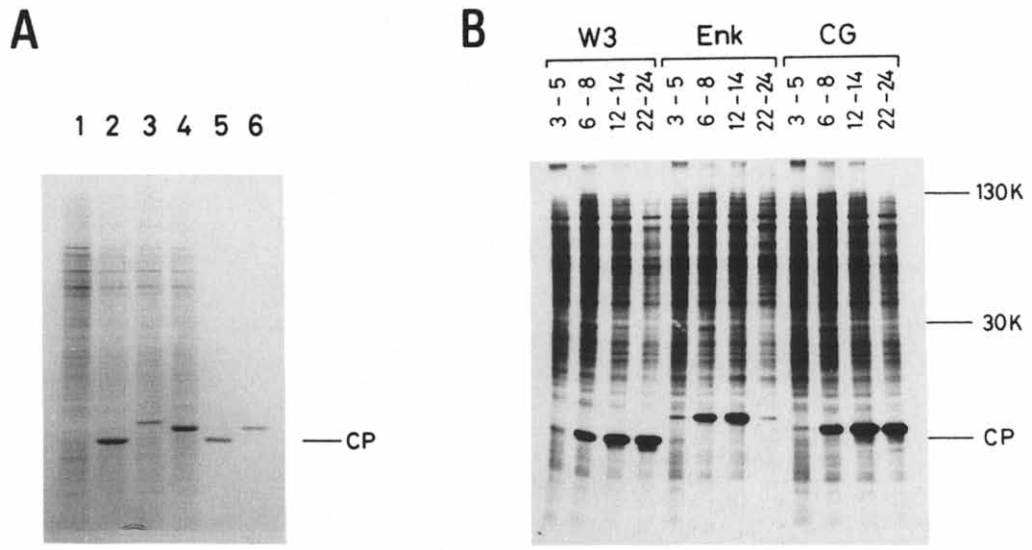

Fig. 3. Analysis of protein produced in protoplasts inoculated with W3, ENK or CG RNA. (A) Gel electrophoresis showing accumulation of CP and its derivatives in the inoculated protoplasts. 10^6 protoplasts were inoculated with approximately 2 μg of W3 RNA (lane 2), ENK RNA (lane 3), CG RNA (lane 4) or mock-inoculated (lane 1), and the lysates of 10^4 protoplasts at 24 h post-inoculation were subjected to NaDodSO$_4$/12% polyacrylamide gel electrophoresis, followed by staining with Coomassie blue. In parallel lanes, 0.3 μg each of the particle proteins of viruses purified from the inoculated leaves of the systemic plants inoculated with W3 RNA (lane 5) or CG RNA (lane 6) were loaded. (B) Fluorogram showing the protein synthesis profiles. Electroporation was carried out as above and proteins were labeled by adding [^{35}S]methionine to the medium for two hours starting 3, 6, 12 or 22 h post-inoculation as indicated above the lanes. Electrophoresis was performed as above and the labeled proteins were detected by fluorography. The positions of the 130 kDa, 30 kDa and coat proteins are indicated in the margin.

RNA (Fig. 3A, lanes 5 and 6). On the other hand, ENK RNA showed no systemic mosaic symptoms upon inoculation onto *N. tabacum* L. cv. Samsun even at two weeks post-inoculation and produced local lesions on the inoculated leaves (Fig. 2). To examine whether virus particles were formed in the case of ENK RNA, back-inoculation was carried out by using the homogenate of the local lesions as inoculum. Unlike W3 RNA, no local lesions were produced and thus, in the case of ENK RNA, no virus particles appear to be formed.

3.3. *Protein synthesis in tobacco protoplasts*

Since ENK RNA caused local lesions even on the systemic tobacco plants, it is difficult to analyze protein synthesis with the whole tobacco plants. Therefore we introduced W3, ENK or CG RNA into tobacco protoplasts by electroporation [17]. The lysates of the inoculated protoplasts at 24 h post-inoculation were subjected to NaDodSO$_4$/12% polyacrylamide gel electrophoresis and stained with Coomassie blue (Fig. 3A, lanes 1–4). Proteins with slower mobility than CP of W3 RNA were accumulated upon inoculation with ENK or CG RNA, and these CP derivative proteins were most abundant in the inoculated protoplasts. The amount of CP derivative of CG RNA was similar to that of CP of W3 RNA, whereas the amount of CP derivative (CP + Enk) of ENK RNA was about three-tenths that of CP of W3 RNA (Fig. 3A, lanes 2–4). The amount of CP (158 amino acids) of W3 RNA was about 0.5 μg per 10^4 protoplasts (Fig. 3A, lanes 2 and 5). In addition, CP derivative of CG RNA had the same mobility as that of the virus particle protein purified from the plants inoculated with CG RNA (Fig. 3A, lanes 4 and 6). Further analysis was done by labeling protoplasts with [^{35}S]methionine (Fig. 3B). All three RNAs showed similar profiles of protein synthesis. CP or its derivatives were detected by labeling from 3 to 5 h post-inoculation and the 30 kDa and 130 kDa proteins encoded by TMV RNA could be detected by labeling from 6 to 8 h post-inoculation. In the case of ENK RNA, however, synthesis of CP + Enk was reduced during 22–24 h post-inoculation.

To confirm the amino acid sequence of the Enk portion of CP + Enk, lysate was prepared from 10^6 protoplasts inoculated with approximately 2 μg of ENK RNA at 24 h post-inoculation and fractionated by NaDodSO$_4$/12% polyacrylamide gel electrophoresis. By elution from the gel band, approximately 0.4 nmol of CP + Enk was recovered. BrCN-treated CP + Enk contained a peptide showing the same retention time on a C_{18} reverse phase column as that of the authentic Enk (see section 2). Such a peptide could not be detected with BrCN-treated CP (data not shown). The amino acid sequence of this peptide was confirmed to be Tyr-Gly-Gly-Phe-Leu, that of Enk (data not shown).

4. DISCUSSION

To examine the validity of the strategy of expressing a foreign gene as a fusion protein with CP using a TMV RNA vector, we constructed ENK and CG RNAs. Both CP derivatives were produced as the major protein in

inoculated protoplasts (Fig. 3A, lanes 3 and 4). Thus, at least in the case of small peptides, it is possible to express a peptide as a fusion protein with CP in large quantity. Virus particle formation was observed with CG RNA but not with ENK RNA. The observation with CG RNA, however, indicates that in some cases the fusion protein may be easily purified as virus particles from the inoculated tobacco plants. In addition, virus particle formation is likely to enhance the stability of CP derivatives since CP derivative of CG RNA accumulated to a higher level than that of CP derivative of ENK RNA (Fig. 3A, lanes 3 and 4).

It is of interest that ENK RNA produced local lesions on the inoculated leaves of *N. tabacum* L. cv. Samsun and the pathogenesis-related proteins [21] were induced in the inoculated leaves (data not shown). *N. tabacum* L. cv. Samsun is devoid of both the *N* and *N'* genes which are considered to be responsible for local lesion formation [22, 23]. Comparison of proteins or mRNA in ENK RNA-inoculated leaves between *N. tabacum* L. cvs. Xanthi nc with the *N* gene and Samsun without the *N* gene may give a clue to the identity of the *N* gene.

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