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Generation of Antiserum Reactive with the 30K Movement Protein of the Tobacco Mosaic Virus Rakkyo Strain

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Summary

Antiserum reactive with the 30K movement protein of the tobacco mosaic virus rakkyo strain was generated for specific detection of the protein. The DNA region encoding the C-terminal portion of the TMV-R 30K protein was fused downstream to the *MalE* gene in pMAL-c2. A fusion protein (MBP-30K) composed of the maltose-binding protein and the C-terminal 163 amino acids of the TMV-R 30K protein was overproduced in *Escherichia coli*. The MBP-30K fusion protein was purified by SDS-polyacrylamide gel electrophoresis and used for immunizing rabbits to generate antiserum. The generated antiserum reacted specifically with the TMV-R 30K protein from infected protoplasts and intact leaves in Western blotting.

Key words: Tobacco mosaic virus, rakkyo strain, 30K protein, antiserum, expression.

Introduction

The tobamoviruses are rod-shaped viruses with single-stranded, positive-sense RNA genome of about 6.4 kb. It encodes at least four proteins: the 130K and 180K proteins, involved in viral replication processes; the 30K protein, required for cell-to-cell movement of the virus; and the coat protein (CP), involved in long-distance spread of the virus as well as in the assembly of virus particles. The 180K protein results from the readthrough at the amber termination codon of the 130K protein[12]. Recently, a rakkyo strain of tobacco mosaic virus (TMV-R), which exhibits distinct host range and symptomatology differences from the common strain of TMV, was described[7].

In this study, to facilitate the analysis of possible roles played by the 30K movement protein of TMV-R in determining its unique biological features, we established a system that overproduces a fusion protein composed of maltose-binding protein (MBP) and the C-terminal portion of TMV-R 30K protein, and generated antiserum that reacts specifically with the 30K protein of TMV-R.

Materials and Methods

Plasmid construction and isolation. pTMR 2-5 (a previously generated clone)[8] and pMAL-c2 (New England BioLabs) plasmid DNAs were restricted with *Bam*HI and subjected to agarose gel electrophoresis. Desired DNA fragments were recovered from the gel using Gene Clean II kit (BIO 101) according to the manufacturer's recommendations. The two purified fragments were then ligated and transformed into XL 1-Blue (Strategene) *Escherichia coli* cells by heat shock at 42 °C for 2 min. Plasmid DNA isolation was carried out by the boiling method[6]. Recombinant plasmid (pTA-MP1) was screened by restriction map and confirmed by sequence analysis.

Expression and purification of MBP-30K fusion protein. One hundred ml of LB medium with 50 mg/ml ampicillin and 12.5 mg/ml tetracycline was inoculated with 2 ml of overnight culture of XL 1-Blue cells containing pTA-MP1. The mixture was incubated at 37 °C with shaking at 165 rpm to an OD_{600} of 0.5. The expression of the MBP-30K fusion protein was induced for 5 hr with the addition of 3 mM isopropylthio-β-D-galactoside (IPTG). For the purification of expressed MBP-30K fusion protein, culture cells were subjected to sonication[14] and lysozyme treatment[2]. Culture cells were collected by gentle centrifugation and resuspended in 0.01 M phosphate buffered saline (PBS) containing 2% Triton X-100 (pH 7.4) and lysed for 10 min using a probe sonicator with a 2 mm diameter probe (output 1; duty cycles 40%) (Branson). After insoluble protein was collected, the mixture was lysed at room temperature in lysis buffer (20 mM Tris-HCl, pH 8.2, 8% sucrose, 100 mM KCl, 5 mM EDTA, 0.1% Nonidet P-40 and 2.5 mg/ml lysozyme). The viscosity of the lysate was reduced by the addition of 10 mM $MgCl_2$ and 10 mg/ml DNase I. The insoluble fraction containing MBP-30K fusion protein was collected by centrifugation. The pellet was resuspended and washed 5 times in lysis buffer without lysozyme. The insoluble protein was disrupted by the addition of an equal volume of Laemmli sample buffer[9] and heated for 5 min in boiling water before electrophoresis. The boiled mixture was subjected to electrophoresis in 10% SDS-polyacrylamide gels as described by Laemmli[9]. The gel was soaked in cold 0.25 M KCl solution and the band of MBP-30K fusion protein was cut out by a razor blade. Protein was eluted from gel by homogenizing the gel with a Teflon homogenizer in PBS (pH 7.4) and centrifuged. The supernatant containing MBP-30K fusion protein was used for immunization.

Generation of antiserum. Antiserum against MBP-30K fusion protein was produced in rabbits by 5 intramuscular injections of purified MBP-30K fusion protein emulsified with Freund's complete adjuvant (1st injection) and incomplete (2nd and following injection). Antiserum was collected from injected rabbits 2 weeks after the final injection. Preimmune normal serum was collected from each rabbit before the first injection.

Examination of antiserum titers. Indirect-ELISA was used to monitor the titers of antiserum. Total soluble protein extracts from induced bacterial cells were coated onto

polystyrene microtiter plate (Nunc Intermed) in carbonate buffer (pH 9.6) for 4 hr at 37°C. After washing with 20 mM phosphate-buffered saline (pH 7.4) (PBS) containing 0.05% Tween 20 (PBS-T), antiserum collected from injected rabbits were serially diluted with PBS (pH 7.4), applied to the plate, and incubated overnight at 4°C. Alkaline phosphatase conjugated anti-rabbit IgG was then added and incubated at 37°C for 4 hr. Finally, 1 mg/ml p-nitrophenylphosphate in 10% diethanolamine (pH 9.8) was added and incubated for 1 hr at 25°C. The absorbance values at 405 nm were recorded using Easy Reader 400FW (SLT Labinstruments).

Protoplast inoculation and extraction of 30K protein from infected tobacco protoplasts and intact leaves. Suspension culture cells of the BY (BY-2 cell line) were maintained, and protoplasts were isolated and electroporated with TMV-R RNA essentially as described[16,17]. Cells were collected by gentle centrifugation and lysed in Laemmli sample buffer[9]. 30K protein from infected tobacco leaves was extracted as described[10]. Samples containing the 30K protein from protoplasts and tobacco leaves were subjected to electrophoresis in gels of 10% polyacrylamide containing SDS.

Western blotting. Western blotting was conducted essentially as described[3]. Briefly, after SDS-PAGE electrophoresis, proteins were subsequently transferred onto Immobilon PVDF membranes (Millipore) with a Trans Blot apparatus (Bio-Rad). After blocking with 5% non-fat dried milk in Tris buffered saline-Tween (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20), the membranes were incubated overnight at 4°C in Tris buffered saline -Tween containing 1% non-fat dried milk and 5 μg/ml of rabbit IgG which was prepared from antiserum against MBP-30K fusion protein. The membranes were washed three times and then incubated with horseradish peroxidase (HRP)-goat anti-rabbit IgG conjugate (ZYMED) for 3 hours at 37°C. Immunoreactive protein bands were detected with Konica Immunostaining HRP-1000 as recommended by the manufacturer.

Results and Discussion

Construction of plasmid encoding MBP-30K fusion protein. The strategy for construction of plasmid encoding the fusion protein is shown in Fig. 1. Recombinant plasmids with the correct orientation were screened by restriction map and confirmed by sequence analysis using a specific oligonucleotide. One clone designated pTA -MP1 was selected for further study. This construction should result in the expression of a fusion protein composed of MBP and the C-terminal portion (from aa 106 to

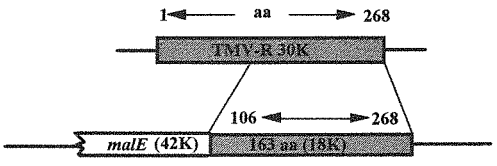

Fig. 1. Strategy for expressing of the C-terminal region of TMV-R 30K protein in *E. coli* as a fusion protein to maltose-binding protein. DNA region encoding the C -terminal 163 amino acids of TMV-R 30K protein was fused downstream to the *malE* gene in pMAL-c2.

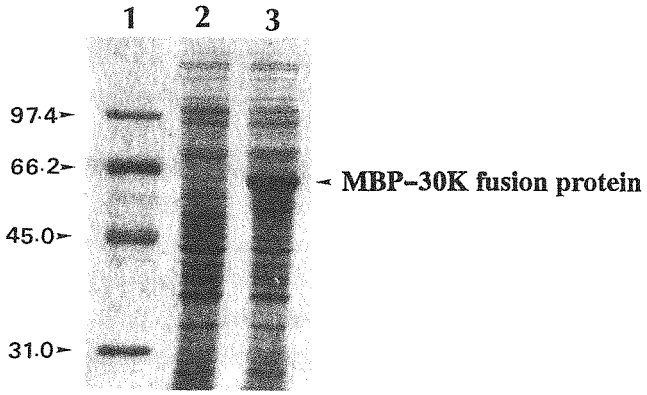

Fig. 2. SDS-PAGE analysis of the expressed fusion protein in *E. coli*. Total proteins from *E. coli* harboring the recombinant expression vector were subjected to electrophoresis in gels of 10% polyacrylamide containing SDS and stained with Coomassie brilliant blue. Lane 1, molecular weight marker in kDa; lane 2, total proteins from uninduced cells; lane 3, total proteins from induced cells.

268) of the 30K protein of TMV-R (Fig. 1).

Overexpression of MBP-30K fusion protein in *E. coli*. *E. coli* carrying pTA-MP1 were grown until the late log phase and then IPTG was added to the culture to induce expression of the fusion protein. Five hours after induction, the total cellular proteins were extracted and analyzed by SDS-PAGE (Fig. 2). A protein band with the expected molecular weight of 60 kDa corresponding to the MBP-30K fusion protein was detected from induced but not from uninduced bacterial cells. This protein was also not detected from induced bacterial cells that was not transformed with pTA-MP1 (data not shown). Pilot experiments were conducted to determine whether the MBP-30K fusion proteins presented in bacterial cells as soluble proteins or as insoluble inclusion bodies. The results showed that the MBP-30K fusion proteins were present in both forms in induced bacterial cells, each represented approximately 50% of the total expressed proteins.

Antiserum raised against MBP-30K fusion proteins. The insoluble MBP-30K fusion proteins from inclusion bodies were purified as described. Rabbits were immunized with fusion proteins eluted from SDS-PAGE. Titers of antiserum were examined by I-ELISA using total soluble proteins from induced bacterial cells after the third injection. Although antiserum titers from the 2 injected rabbits were almost the same, we found that the preimmune serum collected from one rabbit reacted strongly with the total soluble proteins from induced bacterial cells (Fig. 3). Thus, the antiserum with lower background reaction was used in further study.

Detection of TMV-R 30K protein. TMV-R-infected and mock-infected tobacco protoplasts were harvested at 22 hours post-inoculation. Proteins were lysed by boiling the

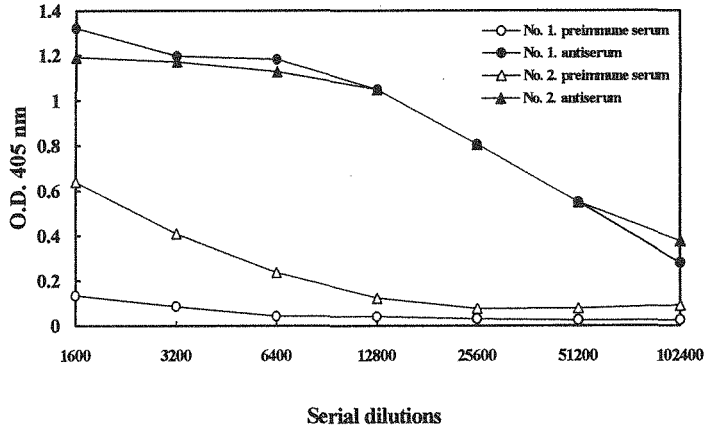

Fig. 3. Examination of antiserum titers by I-ELISA. Antiserum and preimmune normal serum were serially diluted and their reactivities with total soluble extracts from induced bacterial cells containing the MBP-30K fusion protein were tested by I-ELISA. Antiserum and preimmune serum from two rabbits (No. 1 and No. 2) were tested.

protoplasts in Laemmli buffer, separated by SDS-PAGE, and detected by Western blotting using antiserum to the MBP-30K fusion protein (Fig. 4). As expected, the TMV-R 30K protein was specifically detected from extracts of TMV-R infected protoplasts with antiserum to the MBP-30K fusion protein. The anti-fusion protein antiserum did not react with proteins from mock-infected protoplasts. Similar results were obtained from TMV-R infected tobacco leaves (data not shown).

Many plant viruses encode the function for their own spread in plants. The 30K protein of tobacco mosaic virus has been demonstrated to be involved in cell-to-cell movement by reverse genetical analysis of the 30K protein gene[11] and biological analysis of transgenic plants expressing the 30K protein[5]. It has also been shown that the 30K protein enlarges the plasmodesmatal exclusion limit between cells in transgenic plants[18] and also binds preferentially to single-stranded nucleic acids *in vitro*[4]. Immunocytological localization has revealed that the 30K protein accumulates in the plasmodesmata of TMV-infected tobacco plants[15] and in transgenic plants expressing the 30K protein[1,13]. In addition to its role in virus transport, the 30K protein may be involved in symptomatology and in determination of host specificity. The generated antiserum that reacts specifically with the 30K protein of TMV-R would be very useful for the

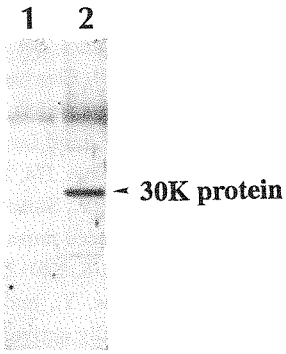

Fig. 4. Detection of TMV-R 30K protein from infected protoplast by Western blotting using antiserum to the MBP-30K fusion protein. Total proteins from an equivalent of 10^4 protoplasts were analyzed by Western blotting. Lane 1, total proteins from mock-inoculated protoplasts; lane 2, total proteins from TMV-R infected protoplasts.

analysis of possible roles played by the 30K movement protein of TMV-R in determining its unique biological features.

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タバコモザイクウイルス・ラッキョウ系統の30K移行タンパク質に対する抗血清の作製

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摘　　要

TMV－Rの30K移行タンパク質を検出するために、本タンパク質に対する特異的な抗体を作製した。まず30KタンパクのC末端領域(163アミノ酸)をコードするDNAをプラスミドpMAL-c2に組み込み、maltose－bindingタンパク質との融合タンパク質として、大腸菌で発現させた。その融合タンパク質をSDS－ポリアクリルアミドゲルより抽出して、ウサギを免疫し、抗血清を作製した。本抗血清を用いて、Western blottingを行ったところ、TMV－Rが感染したタバコプロトプラストおよびタバコ葉から30Kタンパク質が特異的に検出された。