Rapid Diagnosis of Chinese Yam Necrotic Mosaic Virus Infection by Electro-Blot Immunoassay

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Abstract

Chinese yam necrotic mosaic virus (CYNMV) was purified from naturally infected chinese yam leaves by repeated differential centrifugation. The molecular weight of the capsid protein was determined as 38,000 (38K) with SDS-polyacrylamide gel electrophoresis. The antiserum produced against purified CYNMV preparation had a titer of 1:4,096 as determined by immunosorbent electron microscopy and was used for the detection of the 38K polypeptide from leaf homogenate by electro-blots immunoassay. The procedure was simplified to complete all the steps within 7 hours. The 38K polypeptide was readily detected from diseased leaf homogenate diluted up to 1/1,000 but not from healthy leaf homogenate. Reaction with host plant proteins was reduced by diluting the antiserum up to 1/5,000 without losing sensitivity to CYNMV capsid protein.

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Key words: Chinese yam necrotic mosaic virus, electro-blot immunoassay.

Introduction

Chinese yam necrotic mosaic virus (CYNMV) has occurred commonly on chinese yam plants (Dioscorea batatas Decne.) grown in Japan and causes mosaic and necrotic line patterns on the leaves accompanied by stunting of the whole plants, resulting in 10-50% loss in the yield of tubers. The causal virus has a flexuous, rod-shaped particle 660 nm in length and is transmitted by aphids. It is thus considered a member of the carlavirus group. The virus infection has been diagnosed by mechanical inoculation or aphid transmission to Dioscorea spp. plants or electron microscopy of leaf-dip preparations. Serological diagnosis has not been established because of difficulty in preparing the virion antigen with enough quantity and purity due to viscous glycoproteins in the leaf homogenates. In this paper, we describe a procedure for detecting CYNMV capsid protein from leaf homogenates by electro-blots immunoassay with the antiserum against purified CYNMV particles. The procedure was simplified from the original methods and requires less than 7 hours, including time for preparing samples and SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Materials and Methods

Materials. Diseased chinese yam leaves with typical symptoms were collected from

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an experimental field in Aomori Field Crops and Horticultural Experiment Station in the summer of 1985. Leaves without any visible disorder obtained from a meristem-cultured line were tentatively considered as healthy. Nitrocellulose sheet was obtained from Toyo Roshi, alkaline phosphatase-conjugated anti-rabbit IgG goat IgG was from Tago, and nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt (BCIP), were from Sigma.

**Virus purification.** Diseased chinese yam leaves were homogenized in 0.1 M sodium borate, pH 8.0, 1 mM EDTA (3 ml per g tissue) in a Waring blender and centrifuged at 12,000×g for 10 min in a Hitachi RPR 12 rotor. The supernatant was clarified by addition of 2% Triton X-100 and was centrifuged at 156,000×g for 60 min through a pad of 6 ml of 20% sucrose per tube in a Hitachi RP 50-2 rotor. The pellet was suspended in 0.1 M sodium borate, pH 8.0, 1 mM EDTA. The low speed and high speed centrifugations were repeated four times and the final pellet was suspended in water.

**Antiserum production.** The purified virus suspension was injected into a rabbit intramuscularly with Freund complete adjuvant three times at two-week intervals. The serum was collected two weeks after the last injection. The titre was determined by immunosorbent electron microscopy (ISEM) as described by Yanagida and Ahmad-Zadeh (1970).13)

**Sample preparation for SDS-PAGE.** Leaf homogenate prepared by grinding 0.1 g of leaf tissue in 1 ml of 1×sample buffer (SB) (50 mM Tris-HCl, pH 8.8, 2% SDS, 1% mercaptoethanol) was heated at 100°C for 5 min and clarified by centrifugation at 6,000×g for 5 min in a Tomy TMS-1 rotor. The supernatant was considered as a 1/10-diluted leaf homogenate. A concentrated preparation was obtained from the pellet after the first high speed centrifugation step of the virus purification procedure given above. The pellet was suspended in 1 ml of water per 10 g leaf tissue. The purified preparation was from the fourth high speed pellet, suspended in 1 ml of water per 50 g leaf tissue. After adding equal volumes of 2×SB, the concentrated and purified preparations were heated at 100°C for 5 min.

**SDS-PAGE.** Samples of 20 µl per lane of the leaf-homogenate, concentrated and purified preparations were electrophoresed in a 10% polyacrylamide slab gel (14 cm in width, 10 cm in length and 0.1 cm in thickness) at 25 mA, constant current, for 2 hr in the Atto model SJ-1060SD apparatus, with the buffers described by Laemmli (1970).4)

**Electro-blotting.** Polypeptides separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose sheet as described by Towbin et al. (1979)9) at 5 V/cm, constant voltage, for 1 hr using Marysol model KS 8440 II apparatus.

**Enzyme-linked immunoassay.** After electro-blotting, the nitrocellulose sheet was treated as described by Hibi and Saito (1985)31 and Leary et al. (1983)5) with a few modifications. All the steps were conducted at room temperature in a single plastic container with gentle shaking. The blotted sheet was rinsed in 20 mM Tris–HCl, 0.15 M NaCl, 0.05% NaN3, pH 7.5, containing 0.05% Tween 20 (TBST) for 15 min and then incubated in CYNMV antiserum diluted at 1/2,000 with TBST for 30 min. The sheet was washed three times for 5 min per wash in TBST and treated with alkaline phosphatase-conjugated anti-rabbit IgG goat IgG diluted at 1/1,000 in TBST for 30 min. The sheet was washed twice in TBST for 5 min and once in 0.1 M Tris–HCl, 0.1 M NaCl, 5
mM MgCl₂, pH 9.5 (AP9.5) for 5 min. Substrate solution was prepared by mixing 6 ml of 0.033% (W/V) NBT in AP9.5 and 20 µl of 5% (W/V) BCIP in dimethylformamide. NBT and BCIP stock solutions were individually stored at 4°C in the dark. The nitrocellulose sheet was incubated in the substrate solution for 10 min or longer and then washed in water to terminate the enzyme reaction. Usually enough color development was obtained within 10 min.

Results

Virion properties
Electron microscopic observation of the purified CYNMV preparation stained with 2% potassium phosphotungstate, pH 6.5, revealed flexuous, rod-shaped particles 660 nm in length, which corresponds to the value reported by Fukumoto and Tochihara (1978). With SDS-PAGE, a single polypeptide with molecular weight (MW) of 38,000 (38K) was detected from the preparation (Fig. 1) and it was considered to be the CYNMV capsid protein.

Electro-blot immunoassay
The titer of the CYNMV antiserum was 1:4,096 as determined by ISEM. The antiserum was used for the detection of the 38K polypeptide by electro-blot immunoassay by the procedure summarized in Fig. 2. Fig. 3A shows Coomassie brilliant blue R-stained polypeptide patterns after SDS-PAGE. The position of CYNMV capsid protein is shown in lane 1. The concentrated preparations from healthy and diseased leaves contained many species of polypeptides in common but the preparation from the diseased material (lane 3) included the 38K polypeptide, CYNMV capsid protein, which was lacking in that from the healthy material (lane 2). The polypeptide patterns derived from the leaf homogenates of healthy and diseased leaves were indistinguishable (lanes 4 and 5). An electro-blotted sheet probed with the antibodies and incubated in the substrate solution is shown in Fig. 3B. The amounts of the samples applied were the same as in Fig. 3A. From the purified virus preparation, only the 38K polypeptide had a strong color development (lane 1). The 38K polypeptide was also clearly visible from the concentrated and leaf-homogenate preparations from the diseased leaves (lanes 3 and 5) but not from those from the healthy leaves (lanes 2 and 4). The polypeptides other than 38K detected in the concentrated preparation should be the host plant proteins that were preferentially
sedimented by high speed centrifugation. From the leaf homogenate, a series of polypeptides were detected from the top to the bottom of the lane for both healthy and diseased samples but the presence of the 38K polypeptide in the diseased leaf homogenate was distinct.

**Effects of diluting the antiserum and the homogenate on the appearance of the 38K polypeptide and host plant protein**

In Fig. 4A, CYNMV antiserum was diluted from 1/100 to 1/500,000. The amount of the sample applied in each lane was 20 µl of 1/10-diluted, diseased leaf homogenate. The color development was stopped in 10 min. When less diluted antiserum such as 1/100 or 1/200 was used, strong color development appeared on host plant proteins and interfered with the appearance of the specific 38K polypeptide. However, with the antiserum diluted at 1/1,000 to 1/5,000, non-specific reactions were reduced significantly without losing the sensitivity to the 38K polypeptide. The 38K polypeptide was clearly visible even with 1/500,000-diluted antiserum on the original sheet. On the other hand, in Fig. 4B, the sample applied in each well was diluted from 1/10 to 1/50,000. The dilution of CYNMV antiserum was 1/2,000. The color development was continued for 30 min. Although the amount of the sample and the antiserum dilution was identical in lane 5 of Fig. 4A and in lane 1 of Fig. 4B, the latter gave much stronger color devel-
Fig. 4. Effects of diluting the antiserum and the leaf homogenate on the appearance of virus antigen and host plant proteins. (A) The antiserum was diluted at (1) 1/100, (2) 1/200, (3) 1/500, (4) 1/1,000, (5) 1/2,000, (6) 1/5,000, (7) 1/10,000, (8) 1/20,000, (9) 1/50,000, (10) 1/100,000, (11) 1/200,000 and (12) 1/500,000. The amount of the samples applied in each lane was 20 μl of 1/10-diluted, diseased leaf homogenate. The sheet was incubated in the substrate for 10 min. (B) The diseased leaf homogenate was diluted at (1) 1/10, (2) 1/20, (3) 1/50, (4) 1/100, (5) 1/200, (6) 1/500, (7) 1/1,000, (8) 1/2,000, (9) 1/5,000, (10) 1/10,000, (11) 1/20,000 and (12) 1/50,000. Twenty μl was electrophoresed in each lane. The dilution of the antiserum was 1/2,000. The sheet was incubated in the substrate for 30 min. The position of the 38K polypeptide was indicated by the arrows.

Discussion

Electro-blot immunoassay\(^1,7,9\) is a powerful method for diagnosing plant virus infection because of its specificity based on two independent properties of the viral structural protein, i.e. the molecular size and the antigenicity, and its sensitivity which is similar to that of ELISA. However, with the original methods, several different buffers must be prepared for the treatments after blotting and it takes two days to complete the procedure. The procedure described in this paper was quick and efficient. Major modifications from the original methods are as follows. First, treating the blotted sheet in TBST for 15 min was enough for preventing non-specific antibody binding. Second, TBST could be used in all the steps except for the final enzymatic reaction. Third, enough binding of the antibodies, both virus specific and enzyme conjugated, was obtained within 30 min, as is also the case for the decoration method of ISEM. Fourth,
the substrates could be stored as stock solutions individually and the working solution prepared by mixing them just before use.

For the practical use of diagnosing plant virus infection, it is a definite requisite that the virus antigen can be clearly detected from leaf homogenates. In order to reduce non-specific reactions with host plant proteins in the homogenate, the antiserum is usually pretreated with plant extracts to remove the antibodies to plant proteins. In this study, we looked for an alternative way to increase the apparent specificity without sacrificing the sensitivity to the virus antigen by examining the effects of diluting the antiserum and the homogenate. From the results in Fig. 4, we conclude that diluting the antiserum to the point where the sensitivity to the virus antigen is not sacrificed would be one of the efficient ways to reduce non-specific reaction with host plant proteins.

An advantage of electro-blot immunoassay is the possibility of detecting two or more virus antigens when they have structural proteins of different MWs, with exactly the same procedure used for a single virus antigen by simply mixing the antisera specific to each virus antigen.10) As yam mosaic virus (YMV),8) a potyvirus naturally infecting yam plants in Japan, has a capsid protein with MW 34K,11) both CYNMV and YMV could be identified simultaneously on a single nitrocellulose sheet.

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Literature cited

からは検出されなかった。健全植物成分との非特異的反応は、抗血清を5,000倍まで希釈することにより、38K蛋白の検出感度を実用上損うことなく、減少させることが可能だった。