日本で発生するジャガイモYウィルスえそ系統に特異的なマウスモノクローナル抗体の作製とその解析

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論　文

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Production and Selection of a Monoclonal Antibody specific to a Japanese Necrotic Strain of *Potato Virus Y*

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Summary

A Japanese necrotic strain of *Potato virus Y* (PVY-T) is serologically related with an ordinary strain of PVY (PVY-O); therefore, a rabbit antiserum against PVY-T that was thoroughly absorbed with the PVY-O antigen has been used for detection of PVY-T. To detect PVY-T more precisely from tobacco leaves or potato young buds by serological methods, I produced monoclonal antibodies specific to PVY-T. A panel of monoclonal antibodies was raised against PVY-T virion using a standard procedure. The binding properties of individual antibodies to PVY-T and PVY-O were analyzed by indirect enzyme-linked immunosorbent assay. Twenty-eight out of 238 hybridoma cell lines obtained in the experiment produced antibodies specific to PVY-T. The 5 T 2 cell line that showed the highest titer among these specific lines was injected back into the mouse peritoneum and ascitic fluid was obtained. The 5 T 2 antibody belongs to the IgG 2 a subclass and has a κ light chain. The nucleotide sequence of the coat protein gene of PVY-T was determined and the 5 T 2 antibody was suggested to react to the N-terminal region of the coat protein.

Introduction

Potato virus Y (PVY) is a type member of the *Potyvirus* genus within the *Potyviridae*, the largest family of RNA plant viruses. PVY infects tobacco plants with various symptoms ranging from vein banding and mild mosaic to severe leaf and stem necrosis depending on strains of the virus. The occurrences of severe necrotic strains of PVY (PVY-N) on tobacco plants have been reported in many countries throughout the world. In Japan, an outbreak of the disease caused by a necrotic strain of PVY was first recognized in 1971, and the disease was once disappeared by renewal of seed potatoes in

the restricted areas. However, the occurrence of the disease was found again in 1981, and the disease has been widespread and has resulted in severe yield and quality reductions of leaf tobacco in Japan. A Japanese necrotic strain of PVY (PVY-T ; it was designated to distinguish a Japanese isolate from the other PVY-N isolates) is serologically related with an ordinary strain of PVY (PVY-O) ; therefore, it is difficult to distinguish PVY-T from PVY-O by serological methods by using polyclonal antibody. In this report, I describe production of mouse monoclonal antibodies specific to PVY-T and the nucleotide sequence of the coat protein gene of PVY-T.

Materials and Methods

Virus strains, virus purification and polyclonal antibody.

PVY-T[1] was used as a Japanese necrotic strain of PVY. PVY-O, PVY-A 1 that causes yellow mosaic symptoms on burley tobacco[2], and PVY-K were used as isolates of the ordinary strain of PVY. All isolates were propagated in tobacco plants and were purified by the method[3] reported for *Watermelon mosaic virus*.

For absorption with PVY-O antigen, γ-globulin was prepared from rabbit antiserum against PVY-T by standard ammonium sulfate precipitation. Equal amount of PVY-O antigen was added to γ-globulin, incubated overnight at 4°C, and antigen-globulin complex was removed by ultracentrifugation. This absorption procedure was repeated three times to obtain absorbed γ-globulin

Immunization of mice, cell fusion and cell culture

Two six-week-old BALB/c mice were immunized by twice intraperitoneal injections with 3 weeks' interval using 100 μg and 50 μg, successively. Three days after last injection, the mice were sacrificed and the spleens were removed aseptically. The B-lymphocyte (2.4×10^7 cells) from the spleens were fused with mouse myeloma cells P 3-X 63-Ag 8.653 (2.0×10^7 cells) using polyethylene glycol. Hybridomas were selected on soft agar plates containing RPMI 1640 medium, hypoxanthine, aminopterin, thymidine, and 20% fetal calf serum[4]. Selected hybridomas were cultured in 2 ml RPMI 1640 medium containing 10 % fetal calf serum in 24-well plates, and each supernatant was assayed for reactivity to PVY-T and PVY-O by indirect enzyme-linked immunosorbent assay (ELISA). Ascitic fluids were produced by intraperitoneal injection of selected hybridomas into mice after repeated cloning of hybridomas on soft agar plates. A part

of the ascitic fluid was subjected to DEAE Sepharose chromatography for purification of IgG. Selected monoclonal antibodies were characterized using MonoAb-ID EIA kit (Zymed).

ELISA

Indirect ELISA and double-sandwich ELISA (DasELISA) were performed as described by Clark and Adams[5]. For indirect ELISA, plates were incubated with purified PVY-T or PVY-O at 10 μg/ml in 0.05 M carbonate buffer, pH 9.6 for 2 hours at 37°C. After washing with phosphate buffer saline containing 0.05% Tween 20, the supernatant of culture medium was incubated for 1 hour at 37°C. Following the incubation with a 1/1000 dilution of alkaline phosphatase-conjugated goat anti-mouse immunoglobulins for 2 hours at 37°C, the plates were incubated with 1 mg/ml of *p*-nitrophenol phosphoric acid for 1 hour at 37°C. For DasELISA, plates were coated with immunoglobulin (at 5 μg/ml for polyclonal antibody or at 0.5 μg/ml for monoclonal antibodies) for 2 hours at 37°C. PVY samples were incubated for 1 hour at 37°C. Following the incubation with alkaline phosphatase-conjugated immunoglobulin (at a 1/1000 dilution of conjugate for an unabsorbed polyclonal antibody or at a 1/10000 dilution of conjugate for monoclonal antibodies) for 2 hours at 37°C, the substrate was incubated for 1 hours at 37°C. The ELISA value, absorption at 405 nm, was measured with a microplate reader .

Cloning of coat protein gene of PVY-T

PVY-T RNA was isolated from purified virus as the method described by Dougherty and Hiebert[6]. Purified viral RNA primed with oligo (dT 12-18) was served as a template for single-stranded cDNA synthesis by reverse transcriptase. Single-stranded cDNA was used as a template for polymerase chain reaction using two oligonucleotide primers (dTTTTGAGCTCACATGTTCTTCACTCCA and dTTTTTCTAGAGGAATGACACAATCGAT ; underlines show restriction sites of *Sac*I and *Xba*I, respectively). After digestion with *Sac*I and *Xba*I, the amplified DNA fragments were cloned into the respective sites of pBluescript II SK(-), and appropriate clones were sequenced using an automated DNA sequencer(ABI, Model 373 A).

Results and Discussion

Characterization of rabbit polyclonal antibody

For evaluation of cross-reactivity of rabbit polyclonal antibody against PVY-T to PVY-O, γ-globulin was purified and absorbed γ-globulin was prepared by three cycles of absorption with purified PVY-O. DasELISA was performed using unabsorbed γ-globulin and absorbed γ-globulin. Fig. 1 shows the results of DasELISA by using unabsorbed or absorbed γ-globulin. Unabsorbed γ-globulin reacted strongly to PVY-O and PVY-A 1, and the ELISA reading of PVY-O at 1000 ng/ml was about half of that of

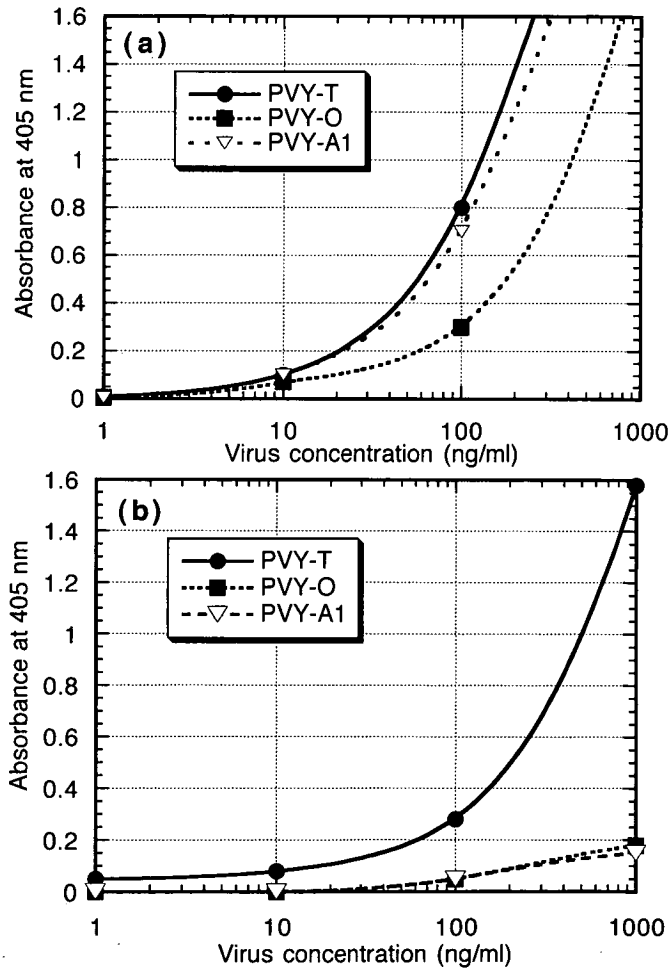

Fig. 1 Comparison of rabbit polyclonal antibody against PVY-T (a) with the absorbed antibody (b) as for reactivity to PVY strains in DasELISA.

PVY-T (Fig. 1 a). Even after three cycles of absorption with purified PVY-O, absorbed γ-globulin still had reactivity to PVY-O and PVY-T, and the ELISA readings of both PVY-O and PYY-A 1 strains at 1000 ng/ml were about 1/8 of that of PVY-T (Fig. 1 b). Reactivity of absorbed γ-globulin to PVY-T was greatly reduced by absorption with PVY-O. Five times higher concentration of alkaline phosphatase conjugate was required for absorbed γ-globulin to obtain the same level of the ELISA reading of unabsorbed γ-globulin. These results showed that polyclonal antibody against PVY-T was not sufficient for specific detection of PVY-T ; therefore, I decided to produce monoclonal antibody specific to PVY-T for diagnosis and detection.

Characterization of monoclonal antibodies

From spleen cells of two mice, 238 hybridoma cell lines were isolated from soft agar plates. Of these cell lines, 28 hybridoma cell lines secreted antibodies that reacted positively with PVY-T virion. Absorbance values in indirect ELISA were varied from 0.11 to greater than 2.00 for PVY-T and from 0.00 to 0.32 for PVY-O (Table 1). Three hybridoma cell lines, 1 T 24, 5 T 1 and 5 T 2, producing high titer antibodies to PVY-T were cloned on soft agar plates, and the remaining hybridomas were stored frozen at -80°C. Three hybridoma cell lines secreted immunoglobulins of the IgG 2 a subclass and had κ light chains. The titers of 5 T 1 and 5 T 2 immunoglobulins in ascitic fluids were 10^9 that was 1000 times higher than those secreted in the culture medium (Table 2).

Table.1 Reactions of the culture medium of each hybridoma to PVY-T and PVY-O in indirect ELISA

Cell lines	PVY-T	PVY-O	Cell lines	PVY-T	PVY-O
1 T 22	>2.00[a)]	0.00	5 T 1	>2.00	0.00
1 T 23	0.11	0.02	5 T 2	>2.00	0.08
1 T 24	1.32	0.00	5 T 4	0.11	0.02
2 T 12	0.10	0.04	5 T 16	>2.00	0.00
3 T 10	0.16	0.03	6 T 13	0.15	0.03
3 T 21	0.11	0.00	7 T 2	0.43	0.00
3 T 23	1.00	0.00	7 T 3	0.17	0.00
3 T 24	0.29	0.00	7 T 5	0.81	0.04
4 T 9	0.25	0.00	9 T 12	>2.00	0.00
4 T 15	1.45	0.32	9 T 14	1.68	0.00
4 T 16	0.52	0.02	9 T 16	0.28	0.00
4 T 18	1.35	0.12	10 T 2	>2.00	0.03
4 T 19	0.15	0.00	10 T 3	>2.00	0.15
4 T 22	1.83	0.04	10 T 18	0.03	0.15
			Polyclonal antibody	1.26	0.69

a) Absorbance at 405 nm.

Table.2 Properties of monoclonal antibodies against PVY-T

Cell lines	Culture medium	Ascitic fluid	Subclass	Light chain
1 T 24	10^{5} a)	n.t.a)	IgG 2 a	κ
5T 1	10^{6}	10^{9}	IgG 2 a	κ
5T 2	10^{6}	10^{9}	IgG 2 a	κ

a) Titer in indirect ELISA.
b) Not tested.

Reactivity of 5T1 and 5T2 immunoglobulins

DasELISA was performed for evaluation of cross-reactivity of 5 T 1 and 5 T 2 monoclonal antibodies with other strains of PVY. Among PVY isolates tested, an isolate, PVY-K, that was judged to be the ordinary strain of PVY after inoculation experiment, reacted to both immunoglobulins with different reactivity. The 5 T 2 immunoglobulin

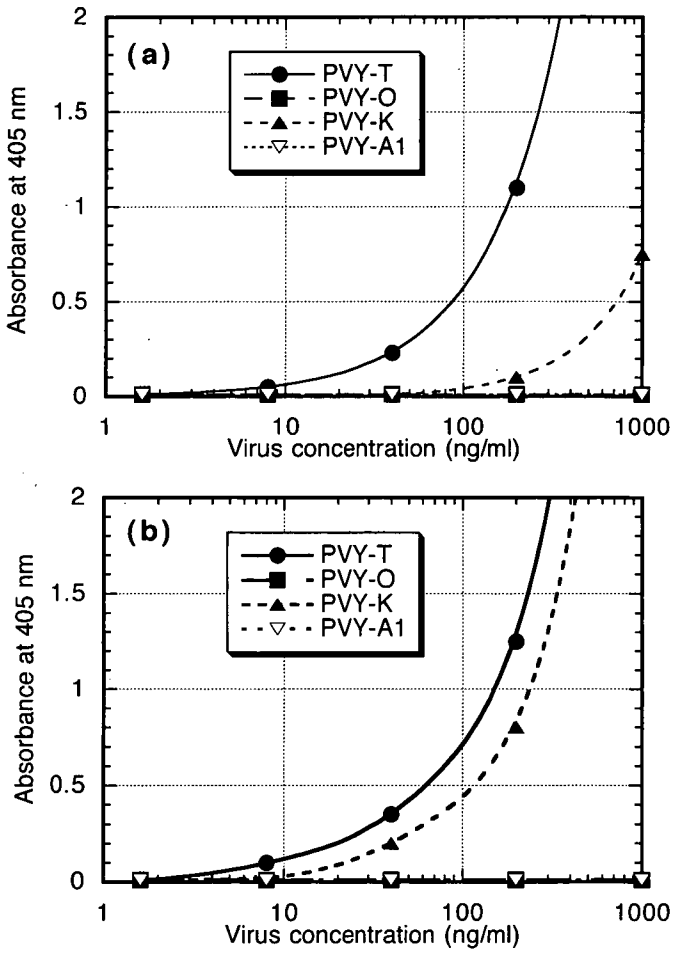

Fig.2 Reactivity of 5 T 1 (a) and 5 T 2 (b) monoclonal antibodies to four isolates of PVY in DasELISA.

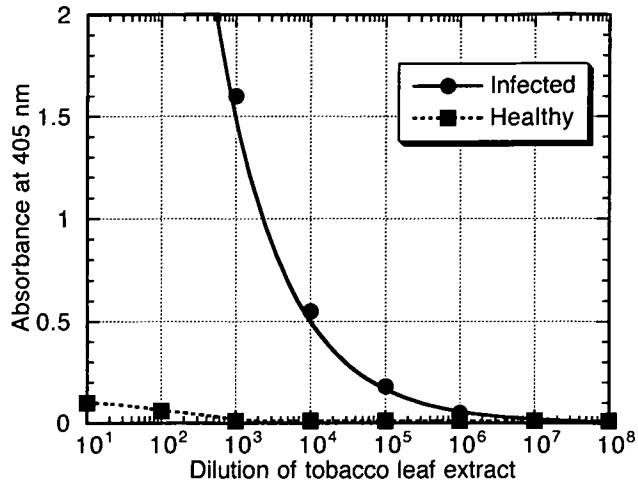

Fig.3 Detection of PVY-T from infected tobacco leaves by DasELISA using a 5 T 2 monoclonal antibody.

had higher reactivity to PVY-K than the 5 T 1 immunoglobulin in DasELISA (Fig. 2 a and Fig. 2 b). This is in line with the report of a necrotic strain specific 4 E 7 monoclonal antibody[7] in which an isolate of the ordinary strain crossreacts to the 4 E 7 monoclonal antibody. Similar results were reported for several other monoclonal antibodies specific to PVY-N[8-12]. These results strongly suggest that the coat protein gene is not a determinant of pathogenicity in tobacco plants. The lower limit of detection of purified PVY-T in DasELISA using the 5 T 2 immunoglobulin was 100 pg/ml, and sensitivity of PVY-T detection from sap of infected tobacco leaves was up to $1:10^6$ dilution of leaf sap (Fig. 3). Slight non-specific reactions were observed around $1:10^2$ dilution of healthy tobacco leaf sap. From these results 5 T 1 and 5 T 2 immunoglobulins were confirmed to be useful for diagnosis of PVY-T. The 5 T 2 antibody has been distributed to tobacco experimental stations for surveillance of potatoes.

Coat protein gene of PVY-T

The coat protein gene of PVY-T was encoded by 801 nucleotides, and the number of predicted amino acid was 267. Percentage of amino acid sequence identity between PVY-T and PVY-O[13] was 91.4%. Fig. 4 shows an alignment of coat proteins of PVY-T, PVY-N[14], and PVY-O. There were only 4 amino acid differences between coat proteins of PVY-T and PVY-N. Amino acid differences between the coat proteins of PVY-T and PVY-O mainly located in the N-terminal region. Thus, it seems probable that the 5T1 and 5T2 monoclonal antibodies recognize some amino acid sequences in the N-

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PVY-T    1  GNDTIDAGGS TKKDVKQEQG SIQPNLNKEK EKDLNVGTSG THTVPRIKAI
PVY-N    1  ---------- ----A----- ---------- V--V------ ----------
PVY-O    1  A------VEI N--ES-P--- ---S-P--G- D--V-A---- ----------

PVY-T   51  TSKMRMPKSK GATVLNLEHL LEYAPQQIDI SNTRATQSQF DTWYEAVQLA
PVY-N   51  ---------- ---------- --------E- ---------- ----------
PVY-O   51  ---------- ---------- ---------- ---------- -------RM-

PVY-T  101  YNIGETEMPT VMNGLMVWCI ENGTSPNING VWVMMDGDEQ VEYPLKPIVE
PVY-N  101  -D-------- ---------- ---------- ---------- ----------
PVY-O  101  ---------- ---------- -------V-- -------N-- ----------

PVY-T  151  NAKPTLRQIM AHFSDVAEAY IEMRNKKEPY MPRYGLVRNL RDGSLARYAF
PVY-N  151  ---------- ---------- ---------- ---------- ----------
PVY-O  151  ---------- ---------- ---------- ------I--- --VG------

PVY-T  201  DFYEVTSRTP VRAREAHIQM KAAALKSAQS RLFGLDGGIS TQEENTERHT
PVY-N  201  ---------- ---------- ---------- ---------- ----------
PVY-O  201  ---------- ---------- ---------P ---------- ----------

PVY-T  251  TEDVSPSMHT LLGVKNM  267
PVY-N  251  ---------- -------  267
PVY-O  251  ---------- -------  267
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Fig.4 Alignment of the deduced amino acid sequence of PVY-T coat protein with those of PVY-N and PVY-O. Identical amino acids are indicated by dashes.

terminal region of the coat protein of PVY-T. If this is true, the coat protein of PVY-K might have similar amino acid sequence in the N-terminal region. In fact, several PVY coat proteins that have similar amino acid sequences in the N-terminal region to those of PVY-N and PVY-T were deposited in protein databanks as isolates of the ordinary strain of PVY. Therefore, we need to keep in mind that a few of the ordinary strains like PVY-K will be inevitably detected even if selected monoclonal antibodies specific to the necrotic strains were used.

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要　約

ジャガイモＹウイルスのえそ系統と普通系統は血清学的に類縁関係があり，ウサギポリクローナル抗体を用いた場合は普通系統で吸収しても，交叉反応の除去が困難である。そこで，えそ系統で免疫したマウスから常法でハイブリドーマ作製して，えそ系統と反応するが普通系統とは交叉反応しないハイブリドーマを選抜して，酵素標識抗体法でえそ系統を検出する手法を確立した。この手法を用いてジャガイモＹウイルス分離株を調べたところ，接種実験で普通系統と判定される分離株の１つがえそ系統特異的モノクローナル抗体と反応し，普通系統とえそ系統の病原性の違いは外被タンパク質によって決定されていないことが示された。