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Serological Properties of Ordinary and Necrotic Isolates of *Potato virus Y*: A Case Study of PVY^N Misidentification

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Abstract In the course of a multi-year survey of *Potato virus Y* (PVY) incidence and diversity in the U.S. seed potato crop, an unusual PVY variant was identified in low but significant levels in multiple states. This variant, PVY^O-O5, was initially detected by a commercially available PVY^N-specific monoclonal antibody, 1F5. This antibody is widely used by U.S. Seed Certification programs to test for PVY^N and is one of two antibodies designated by the North American Plant Protection Organization (NAPPO) for pre-shipment testing of tuber lots that are to be transported between countries. Consequently, PVY^N positives identified by the 1F5 antibody have triggered quarantine actions, prevented cross-border shipments and impacted trade. Here, we demonstrate by a variety of methods that the PVY^O-O5 is a variant within the ordinary PVY strain (PVY^O). Specifically, the PVY^O-O5 variant likely arose due to a single amino acid substitution within the capsid protein. This variant does not induce vein necrosis in tobacco or tuber necrosis in susceptible varieties of potato. Furthermore, it is identified by RT-PCR based diagnostics as PVY^O and it has a typical PVY^O genome sequence. We demonstrate that another PVY^N specific monoclonal antibody, SASA-N, recognizes an epitope distinct from that recognized by 1F5, and correctly identifies the PVY^O-O5 variants as belonging to the PVY^O serotype. Since the PVY^O-O5 variant is present in many seed producing states and misidentification of PVY^O-O5 as $PVY^{N/NTN}$ has clear quarantine implications for export shipments of potato, the limitations of the commercially available monoclonal antibodies should be considered in any certification or phytosanitary testing program.

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Resumen A lo largo del estudio de varios años sobre la incidencia y diversidad del *virus Y de la papa* (PVY) en los cultivos de papa para semilla en los Estados Unidos (EU), se identificó a una variante inusual a niveles bajos pero significativos en múltiples estados. Esta variante, PVY^O-O5, se detectó inicialmente con un anticuerpo monoclonal comercialmente disponible específico para PVY^N, el 1F5. Este anticuerpo es ampliamente usado por los Programas de Certificación de Semilla en los EU para PVY^N, y es uno de los dos anticuerpos designados por la Organización Norteamericana de Protección de Plantas (NAPPO) para pruebas de pre-envío de lotes de tubérculos que serán transportados entre países. Consecuentemente, los PVY^N positivos identificados con el anticuerpo 1F5 han disparado acciones cuarentenarias, evitando envíos trans-fronteras y han impactado al comercio. Aquí, nosotros demostramos con diversos métodos que PVY^O-O5 es una variante del PVY ordinario (PVY^O). Específicamente, la variante PVY^O-O5 es probable que haya surgido debido a una substitución de un amino-ácido dentro de la proteína de la cápside. Esta variante no induce necrosis de las venas en tabaco o necrosis del

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tubérculo en variedades susceptibles de papa. Aún mas, se le identifica como PVY^{O} mediante RT-PCR y tiene la típica secuencia genómica del PVY^{O}. Demostramos que otro anticuerpo monoclonal específico para PVY^{N}, el SASA-N, reconoce un epítope distinto al reconocido por 1F5, e identifica correctamente a las variantes PVY^{O}-O5 como pertenecientes al serotipo PVY^{O}. Tomando en cuenta que la variante PVY^{O}-O5 esta presente en muchos estados que producen semilla, y que la identificación equivocada de PVY^{O}-O5 como PVY$^{N/NTN}$ tiene claras implicaciones cuarentenarias para envíos de exportación de papa, se deberían de considerar las limitaciones de los anticuerpos monoclonales disponibles comercialmente en cualquier programa de pruebas para certificación o fitosanidad.

Introduction

Potato virus Y (PVY) is one of the most economically important pathogens in potato capable of reducing both yield and quality of tubers. PVY exists in nature as a complex of several strain groups and genetic variants within strains (Singh et al. 2008) some of which can be distinguished by symptomatology in different indicator plants, by serological methods, and through molecular genetic analysis. Two broad pathotypes of PVY are defined based on symptoms induced in tobacco. Strains including PVY^{O}, PVY^{C}, PVY^{Z} and PVY^{E} induce mosaic, vein clearing, and mild leaf mottling, while others including PVY^{N}, PVY$^{N\text{-}Wi}$ (syn. PVY$^{N:O}$), and PVY^{NTN} induce systemic vein necrosis. Some (mainly PVY^{NTN} isolates) cause a tuber necrotic reaction in susceptible potato cultivars.

The tobacco necrotic strains of PVY were introduced to North America as early as the 1960s (Kahn and Monroe 1963), although they were not reported from field grown potatoes until the early 1990s (McDonald and Kristjansson 1993), and they have only become widespread in the past decade. Because of this relatively recent introduction and still limited distribution of necrotic strains, management of the ordinary and necrotic pathotypes of PVY has differed in North America potato production. PVY^{O} has been managed primarily by seed potato certification systems relying upon visual symptom ratings during summer field inspections and post harvest testing (visual and limited laboratory testing). The PVY^{C}, PVY^{Z} and PVY^{E} strains are uncommon in North America potato production. The necrotic strains were until recently on the list of quarantine pests for the North American Plant Protection Organization (NAPPO) countries (Canada, Mexico, and U.S.) (Anonymous 2003) and detection often resulted in regulatory actions and suspension of cross-border trade. Numerous reports of necrotic strains present in field grown potatoes (Baldauf et al. 2006; Crosslin et al. 2006; Crosslin et al. 2005; Karasev et al. 2008; Lorenzen et al. 2006a; Nie and Singh 2003; Nie et al. 2004; Piche et al. 2004; Xu 2008; Xu et al. 2005) and the adoption of a US-Canada Binational Tuber Necrotic Virus Management Plan in 2006 has resulted in the removal of necrotic strains of PVY from the quarantine list. Currently the entire PVY complex is managed primarily by seed certification standards. However, the NAPPO potato standards still require that every potato tuber shipment crossing borders between U.S., Canada, and Mexico be tested for PVY$^{N/NTN}$ by ELISA or RT-PCR (Anonymous 2003). The rules specify that ELISA testing has to be done with the use of one of two commercially available monoclonal antibodies, 1F5 or Scottish "Rose". Both of these antibodies were selected because of their specificity to PVY^{N} isolates although it is recognized that there is limited cross-reactivity against some PVY^{O} isolates. Notably, Ellis et al. (1997), reported a PVY^{O} serotype variant labeled PVY^{O5} hereafter referred to as PVY^{O}-O5, which was detected by monoclonal antibody 1F5. Isolates of PVY^{O}-O5 were identified from Manitoba, New Brunswick and Idaho. Additionally, PVY^{O}-O5 isolates were recently reported from New York and Maine (Baldauf et al. 2006). Although 1F5 has been in use on a large-scale in all three NAPPO countries for quarantine purposes, the specificity of this monoclonal antibody, as well as the type of the epitope recognized by 1F5 on PVY particles were poorly understood. In the last few years, the number of cross-border shipments deemed infected with PVY$^{N/NTN}$ by ELISA with 1F5 antibody has increased, prompting a further study of the specificity of this antibody.

Here, we report on the epitope specificities of 1F5 and other PVY-specific monoclonal antibodies and demonstrate that 1F5 antibody binds a conformational epitope on PVY particle. A single amino acid substitution in the capsid protein of PVY^{O}-O5 is likely responsible for this serotype and its reactivity to 1F5. Another PVY^{N}-specific monoclonal antibody, SASA-N, recognizes a PVY^{N}-specific epitope distinct from that recognized by 1F5 and can successfully distinguish between PVY^{O}-O5 and PVY$^{N/NTN}$.

Materials and Methods

Virus Isolates, Polyclonal and Monoclonal Antibodies

All PVY isolates used in this study were from collections shared between labs in Moscow, ID, Aberdeen, ID, and Ithaca, NY. The majority of isolates were collected during a survey of PVY isolates infecting the U.S. seed potato crop from 2004–2006. These isolates were classified to strain using serology, tobacco and potato tuber bioassays, a multiplex RT-PCR assay, and partial or whole genome sequencing. Additional isolates were provided by colleagues.

Polyclonal antisera against PVY PB-Oz, an isolate of PVY^O, were raised in rabbit and in goat following a series of 4–6 immunizations, with the first one in the presence of complete Freund's adjuvant, and all subsequent with the presence of incomplete Freund's adjuvant. The development of the PVY-specific titer was monitored by indirect ELISA, with purified PVY captured on the ELISA plate. Two PVY-specific antisera were produced, UID8 in rabbit, and G500 in goat. Both had good titer exceeding 10^5 in indirect ELISA, with UID8 serum able to detect 1–2 ng/ml of PVY captured onto the plate in indirect ELISA.

Three PVY strain-specific monoclonal antibodies, 4C3 and 1F5 (Ellis et al. 1996; Ellis et al. 1997), and MAb2 (McDonald and Kristjansson 1993), were obtained from Agdia (Elkhart, IN) or from Phyto Diagnostics (North Saanich, BC). Monoclonal antibody 4C3 reacts with PVY isolates belonging to all strains, 1F5 is specific to PVY^N and PVY^{NTN}, and MAb2 is specific to the PVY^O, $PVY^{N\text{-}Wi}$ and PVY^C strains. Two PVY strain-specific monoclonal antibodies, SASA-N and SASA-O, were obtained from Scottish Agricultural Science Agency (SASA, Edinburgh, Scotland). SASA-N is specific to PVY^N and PVY^{NTN}, and SASA-O is specific to the PVY^O, $PVY^{N\text{-}Wi}$ and PVY^C strains. These two antibodies were produced by R. Burns in 1993 and manufactured by SASA for commercial purposes; the SASA-N antibody is distinct from an old monoclonal antibody produced by Gavin Rose (Rose and Hubbard 1986) (C. Douglas, personal communication).

Biological Characterization

The reaction of hosts to isolates of PVY can vary across environments so bioassays in tobacco were carried out in Idaho and New York. Symptoms induced by PVY^O-O5 isolates in tobacco were compared to a set of standard PVY isolates from our lab collections that are maintained in frozen or lyophilized tissue: 423-3 or PB312 (PVY^{NTN}), Alt ($PVY^{N\text{-}Wi}$), and Oz (PVY^O) (Baldauf et al. 2006; Lorenzen et al. 2006a). Virus inoculum was prepared by grinding *ca*. 100 mg of infected tissue (fresh or frozen) in 400 µl phosphate buffer (50 mM sodium phosphate, pH 7.0 plus 20 mM sodium sulfite). Mechanical inoculation was performed using a cotton swab to lightly rub the inoculum on two fully expanded leaves of *Nicotiana tabacum* (cvs Xanthi and Burley) seedlings at the four-leaf stage using carborundum as an abrasive. Each virus isolate was assayed on two test plants of each tobacco cultivar. The plants were grown in an insect-proof greenhouse (NY) or in a growth chamber (ID) under light provided by fluorescent and incandescent lamps with 18 h day/6 h night temperatures of 16°C / 6°C. Symptom observation commenced 2 weeks after inoculation and continued daily for 6 weeks.

To determine if the isolates were capable of inducing potato tuber necrotic ringspot disease (PTNRD) (Beczner et al. 1984), Yukon Gold potato plants grown from cuttings were mechanically inoculated at the 5–6 leaflet stage with each of the isolates described above, five plants per isolate. Plants were tested 2 weeks post inoculation using TAS-ELISA to determine if the mechanical inoculation was successful. The infected plants, planted in 10 cm diameter pots, were grown to maturity in an insect-free greenhouse. The aerial portion of the plant was removed and the soil was allowed to dry for 1–2 weeks prior to harvesting the tubers, which were washed and observed for PTNRD.

Primers, RT-PCR and Sequencing

For multiplex RT-PCR analyses a PVY-Multi 12-primer set described by Lorenzen et al. as used. All steps, including nucleic acid extraction, reverse transcription and subsequent PCR followed the protocol of Lorenzen et al (2006b). Briefly, reverse transcription was performed using 0.8 µl of virus extract in a 15-µl reaction volume that contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 8 mM $MgCl_2$, 1 mM (each) dNTP, 0.12 µM oligo-dT primer mix, 6 units RNase Out Ribonuclease Inhibitor (Invitrogen, Carlsbad, CA), and 60 units of SSII reverse transcriptase (Invitrogen). PCRs were performed in a 20-µl reaction volume that contained 0.8 µl cDNA from above, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.8 mM MgCl2, 0.2 mM (each) dNTP, 0.12 µM for all primers, and 1.0 unit *Taq* DNA polymerase (Promega, Madison, WI). The "touch-down" PCR program consisted of denaturing at 94°C for 2 min, 12 cycles of 94°C for 10 s, 66°C for 30 s (minus 0.5°C per cycle), and 60 s at 72°C, followed by 20 cycles of 92°C for 10 s, 60°C for 30 s, and 72°C for 60 s, ending with a final extension for 7 min at 72°C. PCR products were separated in a horizontal agarose gel and visualized with a fluorescent imager after staining with GelStar (Cambrex, Rockland, ME) or ethidium bromide.

To determine the nucleotide sequence of the CP cistron two sets of primers were used. The first set included universal primers (BlancoUrgoiti et al. 1996) that amplify the entire CP cistron with the exception of the 5′ 23 nucleotides where the primer binds. Since the N-terminus of the PVY CP contains major strain-specific epitopes, a second set of primers was designed to amplify a 443 nucleotide fragment encompassing the 5′ end of the CP cistron based on alignments of over 20 available sequences representing several different strains of PVY. The forward primer 5′-AAGAGCCTTCACTGAAATGATG-3′ starts 72 nucleotides upstream of the CP cistron. The reverse primer 5′-TTCCATTTTCAATGCACCAA-3′ starts 351 nucleotides into the CP cistron. Reverse transcription was performed using 0.6 µl of a total RNA extract in a 15-µl reaction volume using the SuperScriptTM First-Strand Synthesis System (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol. PCRs were performed in a 50-µl

reaction volume that contained 2.0 µl cDNA from above, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.8 mM MgCl2, 0.2 mM (each) dNTP, 0.12 µM for all primers, and 1.0 unit *Taq* DNA polymerase (New England Biolab, Ipswitch, MA). The PCR program consisted of denaturing at 94°C for 2 min, 35 cycles of 94°C for 45 s, 52°C for 60 s, and 60 s at 72°C, ending with a final extension for 5 min at 72°C. The three PVY^{O5} isolates, ID269, ME56, and ME173, from the laboratory collection were maintained in an insect-proof growth room at the University of Idaho. The whole genome sequencing for these three isolates was performed on two large, overlapping RT-PCR amplified fragments generated essentially as described previously (Hu et al. 2009). PCR products purified using the Qiaquick purification kit (Qiagen, Valencia, CA) and sequenced directly using the Applied Biosystems Automated 3730 DNA analyzer with Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase. For multiple alignments CLUSTAL X was used with the default parameters.

ELISA Format and Western Blots

Triple-antibody sandwich (TAS) ELISA tests were performed following the general protocol of Clark and Adams (Clark and Adams 1977) with modifications described previously (Nikolaeva et al. 1997). Wells of Nunc MaxiSorp microtiter plates (Nunc, Rochester, NY) were coated with 200 µl of the G500 antiserum at 1:1,000 dilution in 20 mM sodium carbonate buffer (pH 9.6) and incubated for 4 h at 37°C or 16 to 24 h at 4°C. Plates were washed with 1× PBS buffer containing 0.1% Tween 20 (PBST) and rinsed 3 to 5 times with deionized water, and 200 µl of plant extract was loaded into each well. Plates were incubated with plant extracts for 16 to 20 h at 4°C, washed with PBST, and an intermediate detecting antiserum UID8 at the appropriate concentration was applied to the wells in PBST buffer containing 0.2% bovine serum albumin (BSA). After incubation for 4 h at 37°C (or, alternatively, for 16 to 20 h at 4°C), plates were washed extensively with PBST, and goat anti-rabbit (Sigma A-3687) IgG-conjugates with alkaline phosphatase at 1:30,000 dilution in PBST with 0.2% BSA were added, and the plates were incubated 4 h at 37°C (or 16 to 20 h at 4°C). The plates were washed with PBST, and 0.6 mg/ml of *p*-nitrophenyl phosphate (Sigma) in 0.1 M diethanolamine buffer, pH 9.8, was added as a substrate. The color reaction was monitored by measuring absorbance at 405 nm using an ELISA reader. PVY-positive and PVY-negative potato samples were included into each ELISA experiment as controls. Samples were defined as positive if the absorbance value was 3X the healthy controls.

For the Western blot, leaf samples were collected from tobacco plants infected with PVY isolates, ground in the Laemmli Tris-SDS sample buffer, heated at 95°C for 4 min, and proteins were separated on 4–20% gradient polyacrylamide gels using Laemmli's Tris-SDS protocol (Bio-Rad, USA). Separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad, USA). For Western blotting, the membrane was blocked overnight in 3% dry milk in phosphate buffered saline (PBS) at 4°C. After washing (PBS, 0.1% Tween-20), the membranes were incubated for 2 h, either with the respective monoclonal antibody or with the PVY specific polyclonal antiserum UID8. The membranes were washed (PBS, 0.1% Tween-20) and incubated for 2 h at room temperature with alkaline phosphatase conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Sigma). The immune complexes were revealed by incubating the membranes with BCIP/NBT substrate (Sigma) and the color reaction was stopped by washing them in water.

Results

A total of 154 (5.9%) of the 2,629 PVY isolates collected from the 2004, 2005 and 2006 surveys of the U.S. seed potato crop and characterized by serology and tobacco bioassays were determined to be PVY^{O}-O5 isolates. These isolates were identified from seven of the 16 seed producing states. Just over 20% of the PVY isolates detected from Colorado were PVY^{O}-O5, while between 3–6% of the isolates from Nebraska, Maine, Idaho and Washington were PVY^{O}-O5. Specifically, these isolates reacted with both 1F5 and MAb2 antibodies (see Fig. 1 as an example). Of these, 131 were tested using multiplex RT-PCR and the two amplicons (267 and 689 nt in size) characteristic of the PVY^{O} strain were generated from each of the samples (Fig. 2 as an example). To date, 21 PVY^{O}-O5 isolates from the survey have been tested for their ability to induce PTNRD in Yukon Gold. Typical PTNRD

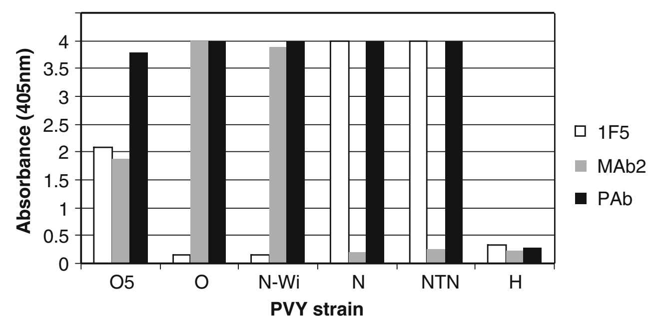

Fig. 1 TAS-ELISA detection of five PVY isolates in tobacco leaf tissue: PVY^{O}-O5 (ID269), PVY^{O}(Oz), $PVY^{N\text{-}Wi}$ (Alt), PVY^{N} (Mont), and PVY^{NTN} (423-3). H = mock inoculated tobacco. All five isolates were captured with the polyclonal goat anti-PVY serum G500 and detected with mouse monoclonal 1F5 (PVY^{N}-specific), mouse monoclonal MAb2 (PVY^{O}-specific), or polyclonal rabbit anti-PVY serum UID8 (PAb)

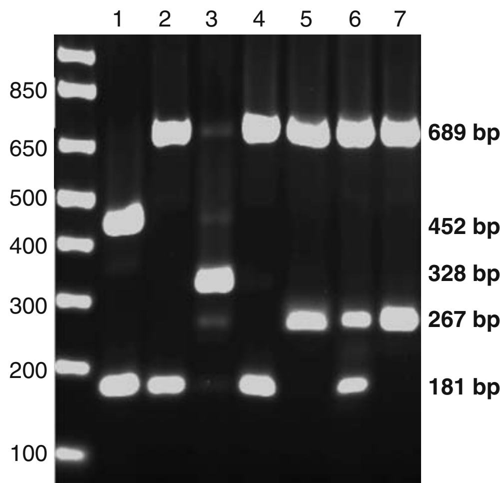

Fig. 2 Differentiation of PVY strains based on the RT-PCR multiplex assay of Lorenzen et al. (2006b). MW = Molecular weight markers, 1 = PVY^{NTN}, 452+181 bp products; 2 = PVY$^{N\text{-}Wi}$, 689+181 bp products; 3 = PVY$^{NA\text{-}NTN}$, 328 bp product; 4 = PVY$^{N\text{-}Wi}$, 689+181 bp products; 5 = PVY^{O}-O5 (ID269), 689+267 bp products; 6 = Mixture of PVY^{O}-O5 and PVY$^{N\text{-}Wi}$, 689+287+181 bp products; 7 = PVY^{O}, 689+267 bp products

symptoms have not been observed on any of the harvested tubers. These biological and serological data are consistent with that reported by Baldauf et al. (2006).

Three PVY^{O}-O5 isolates, ID269, ME56, and ME173, were subjected to a whole genome sequencing (GenBank FJ643477 (ID269), FJ643478 (ME56), FJ643479 (ME173)) and identified as typical PVY^{O}, fully consistent with biological and RT-PCR typing data.

Serological properties of PVY are determined exclusively by the capsid protein and serogroups may be determined by specific recognizable changes in the amino acid sequence. To address effects of possible changes in the capsid protein (CP) sequence, we sequenced the CP cistron from 39 PVY^{O}-O5 isolates collected over four years from seven different states. A low frequency of amino acid substitutions was observed throughout the CP gene in PVY^{O}-O5 isolates of PVY (Fig. 3), but only one substitution a Q to R substitution at position 98 was found to correlate with the observed 1F5-positive reactivity of the PVY^{O}-O5 CP. CP sequences of PVY^{N} or PVY^{NTN} isolates from our collection or from sequences available in the GenBank database indicate that position 98 is occupied by a Q. All the PVY$^{N/NTN}$ isolates from our collection react with monoclonal antibody 1F5. In contrast, position 98 in the CP of isolates belonging either to the PVY^{O} or PVY$^{N\text{-}Wi}$ strains is an R and these isolates do not react with monoclonal antibody 1F5. Thus, this sequence comparison suggested that the Q-98 residue lies within the epitope recognized by the 1F5 monoclonal antibody or has an effect on the structure of the epitope. Neither the location on the PVY CP or the type of the 1F5 epitope has been studied, *i.e.* whether it is a simple, linear epitope or a more complex, conformational epitope (Peter Ellis, personal communication).

To fill this knowledge gap concerning epitope specificity of the PVY-specific monoclonal antibodies, we initially tested the reactivity of different monoclonals to PVY CP in Western blots. Both liquid-phase (ELISA) and solid-phase (Western) data are summarized in

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PVYNTN(PB312)    1-GNDTIDAGGSTKKDAKQEQGSIQPNLNKEKEKDVNVGTSGTHTVPRIKAI-50
PVYO5 (PB945)    1-ANDTIDAGGSNKKDTKPEQSSIQSNLNKGKDKDVNAGTSGTHTVPRIKAI-50
PVYN:O(PB209)    1-ANDTIDAGGSSKKDARPEQGSIQSNPNKGKDKDVNAGTSGTHTVPRIKAI-50
PVYO  (PVY-Oz)   1-ANDTIDAGGSSKKDAKPEQGSIQSNPNKGKDKDVNAGTSGTHTVPRIKAI-50
                                               *

PVYNTN(PB312)   51-TSKMRMPKSKGATVLNLEHLLEYAPQQIDISNTRATQSQFDTWYEAVQLA-100
PVYO5 (PB945)   51-TSKMRMPKSKGAAVLNLEHLLEYAPQQIDISNTRATQSQFDTWYEAVQMA-100
PVYN:O(PB209)   51-TSKMRMPKSKGATVLNLEHLLEYAPQQIDISNTRATQSQFDTWYEAVRMA-100
PVYO  (PVY-Oz)  51-TSKMRMPKSKGATVLNLEHLLEYAPQQIDISNTRATQSQFDTWYEAVRMA-100

PVYNTN(PB312)  101-YDIGETEMPTVMNGLMVWCIENGTSPNINGVWVMMDGDEQVEYPLKPIVE-150
PVYO5 (PB945)  101-YDIGETEMPTVMNGLMVWCIENGTSPNVNGVWVMMDGNEQVEYPLKPIVE-150
PVYN:O(PB209)  101-YDIGETEMPTVMNGLMVWCIENGTSPNVNGVWVMMDGDEQVEYPLKPIVE-150
PVYO  (PVY-Oz) 101-YDIGETEMPTVMNGLMVWCIENGTSPNVNGVWVMMDGNEQVEYPLKPIVE-150

PVYNTN(PB312)  151-NAKPTLRQIMAHFSDVAEAYIEMRNKKEPYMPRYGLVRNLRDGSLARYAF-200
PVYO5 (PB945)  151-NAKPTLRQIMAHFSDVAEAYIEMRNKKEPYMPRYGLIRNLRDMGLARYAF-200
PVYN:O(PB209)  151-NAKPTLRQIMAHFSDVAEAYIEMRNKKEPYMPRYGLIRNLRDVGLARYAF-200
PVYO  (PVY-Oz) 151-NAKPTLRQIMAHFSDVAEAYIEMRNKKEPYMPRYGLIRNLRDVGLARYAF-200

PVYNTN(PB312)  201-DFYEVTSRTPVRAREAHIQMKAAALKSAQSRLFGLDGGISTQEENTERHT-250
PVYO5 (PB945)  201-DFYEVTSRTPVRAREAHIQMKAAALKSAQPRLFGLDGGISTQEENTERHT-250
PVYN:O(PB209)  201-DFYEVTSRTPVRAREAHIQMKAAALKSAQPRLFGLDGGISTQEENTERHT-250
PVYO  (PVY-Oz) 201-DFYEVTSRTPVRAREAHIQMKAAALKSAQPRLFGLDGGISTQEENTERHT-250

PVYNTN(PB312)  251-TEDVSPSMHTLLGVKNM
PVYO5 (PB945)  251-TEDVSPSMHTLLGVKNM
PVYN:O(PB209)  251-TEDVSPSMHTLLGVKNM
PVYO  (PVY-Oz) 251-TEDVSPSMHTLLGVKNM
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Fig. 3 Alignment of consensus amino acid sequences for capsid proteins of PVY^{O}-O5, PVY^{NTN}, PVY$^{N\text{-}Wi}$, and PVY^{O} isolates; all positions with nonsynonomous amino acids are bolded, position #98 of the capsid protein sequence is boxed and shaded in yellow, other nonsynomous amino acids between PVY^{NTN} and the other three groups are boxed and shaded in blue

Table 1 Summary of the antigenic reactivity of the capsid proteins of isolates of PVY representing the different strain groups with different monoclonal antibodies in Western blots and in TAS-ELISA

Isolate (strain)	Western blot			TAS-ELISA		
	1F5[a]	MAb2	SASA-N	1F5	MAb2	SASA-N
ID269 (O5)	−	+	−	+	+	−
PB-Oz (O)	−	+	−	−	+	−
Alt (N-Wi)	−	+	−	−	+	−
Mont (N)	−	−	+	+	−	+
423-3 (NTN)	−	−	+	+	−	+

[a] Monoclonal antibodies 1F5 and SASA-N are reported to be specific for $PVY^{N/NTN}$ strains, MAb2 is reported to be specific for the PVY^{O} strain

Table 1. In Western blots, monoclonal 1F5 did not bind CP from any of the PVY isolates tested, suggesting a conformational epitope for this antibody. This conformational epitope is likely being disrupted during the SDS-denaturation and immobilization steps involved in Western blots. We hypothesize that the Q-98 amino acid residue (Fig. 3) is a part of this conformational epitope. Monoclonals MAb2, and SASA-N, on the other hand, detected the strain-specific PVY CP in Western blots and demonstrate the same specificities as in ELISA, which suggests both these antibodies recognize two distinct linear strain-specific epitopes (see Table 1 and Fig. 4).

Since the 1F5 monoclonal antibody does react with PVY^{O}-O5 isolates and as such is not specific to isolates that induce vein necrosis in tobacco we investigated if another PVY^{N}-specific antibody that recognizes an epitope distinct from 1F5 would be able to differentiate between PVY^{O}-O5 and PVY^{N}/PVY^{NTN} isolates. Based on our Western blot data (Fig. 4), we selected SASA-N monoclonal as a potential candidate since it is available commercially as a PVY^{N}-specific antibody (Scottish Agricultural Science Agency, Edinburgh, Scotland), and it apparently recognizes an epitope distinct from 1F5. Indeed, in a standard TAS-ELISA, identical to the format used for 1F5-based testing, SASA-N antibody only reacted with PVY^{N} and PVY^{NTN}, but not with PVY^{O}-O5, PVY^{O} or $PVY^{N\text{-}Wi}$ isolates (Fig. 5). Furthermore, the PVY^{O}-O5 isolate ID269 did not react with the SASA-N antibody in Western blots (Table 1 and Fig. 4).

In order to test SASA-N on a wider spectrum of PVY^{O}-O5 isolates, we performed TAS-ELISA on a set of 17 of these isolates and 8 PVY^{O} isolates selected randomly from our North American collection. All 25 tests were done in duplicates, side-by-side on the same ELISA plate to avoid plate-to-plate variations. All the 17 PVY^{O}-O5 isolates were correctly identified as PVY^{O} serotypes and all were misidentified as PVY^{N} serotypes with the 1F5 MAb (Table 2).

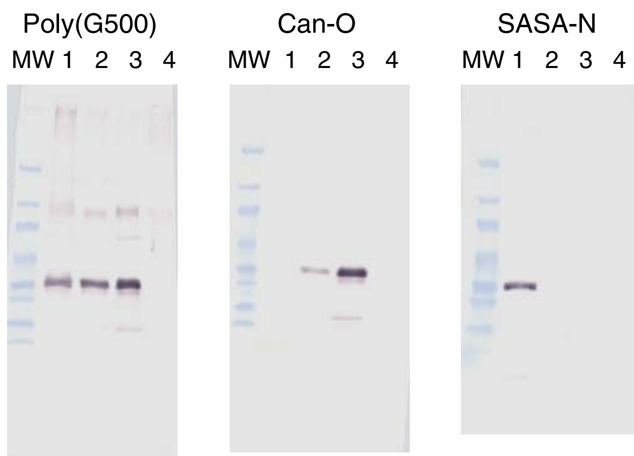

Fig. 4 Western blot analysis of representative PVY isolates extracted from infectedtobacco leaf tissue. MW = molecular weight markers, Lane 1 = PVY^{N} (Mont), Lane 2 = PVY^{O} (Oz), Lane 3 = PVY^{O}-O5 (ID269), Lane 4 = uninfected tobacco tissue. Each blot was separately probed with (A) polyclonal rabbit anti-PVY serum UID8; (B) mouse monoclonal MAb2; or (C) mouse monoclonal SASA-N

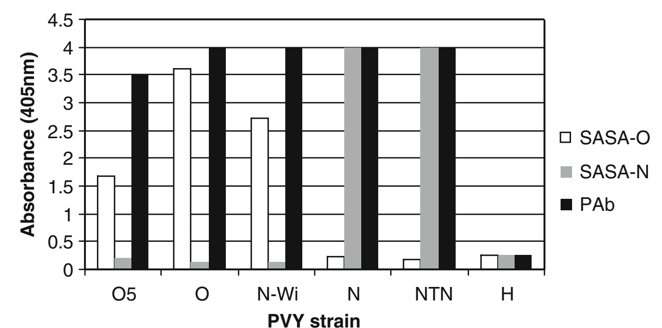

Fig. 5 TAS-ELISA detection of five PVY isolates in tobacco leaf tissue: PVY^{O}-O5 (ID269), PVY^{O} (Oz), $PVY^{N\text{-}Wi}$ (Alt), PVY^{N} (Mont), and PVY^{NTN} (423-3). H = mock inoculated tobacco. All five isolates were captured with the polyclonal goat anti-PVY serum G500, and detected with mouse monoclonal SASA-N (PVY^{N}-specific), mouse monoclonal SASA-O (PVY^{O}-specific), or polyclonal rabbit anti-PVY serum UID8 (PAb)

Table 2 Serotyping and strain typing of various PVY^{O} and PVY^{O}-O5 isolates collected in 2004, 2005 and 2006

Isolate	Year collected	Tobacco symptom		ELISA[a]					Strain[b]	Multiplex RT-PCR[c]
		Mosaic	Vein Necrosis	Pab	Mab2	SASA-O	IF5	SASA-N		
ID1_5_62A	2004	+	−	+	+	+	−	−	O	O
ID11_27_57B	2004	+	−	+	+	+	+	−	O5	O
ID130	2005	+	−	+	+	+	−	−	O	O
ID253	2005	+	−	+	+	+	+	−	O5	O
ID281	2005	+	−	+	+	+	−	−	O	O
ID315	2005	+	−	+	+	+	+	−	O5	O
ID883	2006	+	−	+	+	+	−	−	O	O
ID968	2006	+	−	+	+	+	+	−	O5	O
ID988	2006	+	−	+	+	+	+	−	O5	O
ID1010	2006	+	−	+	+	+	+	−	O5	O
ID1269	2006	+	−	+	+	+	+	−	O5	O
ID243	2006	+	−	+	+	+	−	−	O	O
ID269	2006	+	−	+	+	+	+	−	O5	O
ID331	2006	+	−	+	+	+	+	−	O5	O
ME89-107	2004	+	−	+	+	+	+	−	O5	O
ME286-58	2004	+	−	+	+	+	+	−	O5	O
ME236-4	2004	+	−	+	+	+	−	−	O	O
ME236-77	2004	+	−	+	+	+	+	−	O5	O
ME27	2005	+	−	+	+	+	+	−	O5	O
ME120	2005	+	−	+	+	+	−	−	O	O
ME131	2005	+	−	+	+	+	+	−	O5	O
ME227	2005	+	−	+	+	+	+	−	O5	O
ME200	2006	+	−	+	+	+	−	−	O	O
ME56	2006	+	−	+	+	+	+	−	O5	O
ME173	2006	+	−	+	+	+	+	−	O5	O

[a] Double or Triple antibody sandwich ELISA used a polyclonal antibody (PAb) that recognizes all strains of PVY, monoclonal antibodies 1F5 and SASA-N are reported to specifically recognize the PVY$^{N/NTN}$ strains, monoclonal antibodies PAb2 and SASA-O specifically recognize the PVY$^{O/N\text{-}Wi}$ strains

[b] The strain designation for each of the isolates was based on the data from the tobacco bioassay, ELISA and multiplex RT-PCR

[c] The multiplex RT-PCR assay was described in Lorenzen et al. (2006b) and is able to distinguish the strains of PVY, although it does not distinguish PVY^{O} from the PVY^{O}-O5 variant

Discussion

Necrotic strains of PVY can be an impediment for international movement of potato tuber shipments, both seed and ware. When required, ELISA based testing is a critical component of import testing. For example, current NAPPO standards list ELISA with PVY^{N} and PVY^{O}-specific monoclonal antibodies as one acceptable test, but in reality a majority of the testing conducted by regulatory and seed certification laboratories in NAPPO countries is done utilizing monoclonal antibodies 1F5 (PVY^{N}-specific), and 4C3 (PVY-universal) and therefore the PVY^{O}-O5 isolates will be identified as necrotic isolates. This can trigger regulatory action and prevent movement of potato tubers across borders, despite the fact that PVY^{O}-O5 isolates belong to the benign PVY^{O} strain and should not be subjected to quarantine restrictions for cross-border movement and trade.

The PVY^{O}-O5 isolates are not uncommon and have been reported from four independent surveys done in Canada and the United States since 1996 (Baldauf et al. 2006; Ellis et al. 1996; Ellis et al. 1997; Gray et al. 2008). Isolates with a similar serotype as PVY^{O}-O5, *i.e.* reaction with 1F5 and MAb2 antibodies, were also reported by (Piche et al. 2004), but these are different from PVY^{O}-O5 in that they were determined to be similar to European isolates of PVY^{NTN} using the same multiplex RT-PCR assay employed in this study (Lorenzen et al. 2006b). A recent survey of seed potato acreage in the United States identified PVY^{O}-O5 isolates from seven of 16 seed potato production states and

it was detected at relatively high levels in four of those states (Gray, unpublished). A mixed infection of PVY^{O} and PVY^{NTN} will also generate a serotype identical to PVY^{O}-O5 using the 1F5 and MAb2 antibodies, but these will differ from PVY^{O}-O5 if analyzed by multiplex RT-PCR. Furthermore, the mixed infections will induce vein necrosis in tobacco.

PVY^{O}-O5 isolates are by all tests, except ELISA using monoclonal antibody 1F5, members of the PVY^{O} strain. In this work, we demonstrated that reactivity of the PVY^{O}-O5 isolates to the 1F5 monoclonal antibody is likely due to a single amino acid substitution at position 98 in the CP. The conformational nature of the 1F5 epitope prevents us from defining the exact nature of the epitope by conventional epitope mapping technologies. Reverse genetic studies to mutate position 98 would help, but mutation of other sites within the capsid protein would undoubtedly also affect antibody binding due to the dependence upon specific protein folding. We provide only correlative data indicating that a R to Q change in the PVY^{O} capsid protein is responsible for the generation of the PVY^{O}-O5 variant that has become fixed in the population; however, the correlation holds true across a range of isolates collected in numerous geographic locations and across multiple years. Single amino acid mutations are known to be responsible for shifts in antigenic properties of PVY isolates. Recently, a Syrian PVY isolate, PVY-12, was described with a documented single amino acid substitution in the capsid protein causing a shift in serotype (Ali et al. 2007). PVY-12 belongs to the PVY^{NTN} strain based on various biological and molecular assays, but a single amino acid change from E to G at position 29 (see Fig. 3) allows the virus to be recognized by the PVY^{O}-specific monoclonal antibody MAb2.

It is clear from this and previous studies that serotyping of PVY isolates, while being a convenient typing tool, may not correctly classify them into the strain groups recently proposed by Singh et al. (2008). Additional molecular assays and more importantly, biological assays are required, especially if it is important to determine the pathogenicity of the PVY isolate on tobacco (vein necrosis) or potato tubers (PTNRD). Until the molecular determinants of tobacco vein necrosis and PTNRD are identified, multiple tests will be needed to accurately classify each PVY isolate. This is time consuming and expensive and in most cases not appropriate or necessary for regulatory and seed certification agencies. The PVY^{O}-O5 isolates represent a unique problem that fortunately has a “quick and inexpensive fix”. In regions where PVY^{O}-O5 is known or suspected, it would be prudent to replace the 1F5 antibody as an initial determinant of PVY^{N}/PVY^{NTN} strains with another antibody, e.g. SASA-N that recognizes an epitope distinct from the one recognized by 1F5 and is not affected by the amino acid substitution at position 98 in the PVY CP. This simple amendment in current practices will reduce misidentification of the PVY^{O}-O5 isolates and alleviate regulatory issues and trade sanctions. Furthermore, misidentification of PVY^{O}-O5 that leads to conclusions of existence of necrotic strains of PVY in some areas that are actually free of these strains will lead to wrong assumptions about epidemiology of PVY in a region and, consequently, to erroneous management strategies for addressing the PVY problem.

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