

Epitope Mapping for Monoclonal Antibodies Recognizing Tuber Necrotic Isolates of *Potato Virus Y*

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Abstract *Potato virus Y* (PVY) is an important viral pathogen of potato responsible for reducing tuber yield and quality across the globe. The PVY^N and PVY^{NTN} strains, the latter of which induces potato tuber necrotic ringspot disease (PTNRD), are regulated for international potato trade, and have been routinely detected using monoclonal antibodies (MAbs) that discriminate between PVY^N and PVY^O serotypes. Here, we identify the distinct binding sites in the capsid protein of PVY for three of the four main PVY^N-specific MAbs, Bioreba-N, SASA-N, and Neogen-N, available commercially. These binding domains were mapped through a combination of TAS-ELISA testing of MAbs on multiple reference isolates of PVY, sequence analysis, heterologous expression of capsid protein fragments, and synthetic peptide binding experiments. All three

MAbs were found to bind linear epitopes located within the first 31 N-terminal amino acids of the capsid protein. Bioreba-N MAb epitope spanned aa 1-17 and included three positions, aa 1, aa 11, and aa 17, which differ between PVY^N and PVY^O serotypes. Both SASA-N and Neogen-N epitopes spanned aa 22-30, and included two positions, aa 24 and aa 29, which differ between PVY^N and PVY^O serotypes. Epitopes for SASA-N and Neogen-N MAbs are likely to be identical or overlapping. Examination of available sequences for tuber necrotic isolates of PVY that do not react with PVY^N-specific MAbs SASA-N and Neogen-N indicated possible selection for substitutions in corresponding epitopes leading to the loss of reactivity towards these antibodies. The data obtained suggested that testing with more than one PVY^N serotype-specific MAb could assure a reliable serological identification of a PVY^N or PVY^{NTN} isolate.

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Resumen El virus Y de la papa (PVY) es un patógeno viral importante de la papa responsable de la reducción del rendimiento y calidad de tubérculo en todo el mundo. Las variantes PVY^N y PVY^{NTN}, ésta última induce la enfermedad de la mancha anular necrótica del tubérculo (PTNRD), están reguladas para el comercio internacional de la papa, y se han detectado rutinariamente usando anticuerpos monoclonales (MAbs) que discriminan entre los serotipos PVY^N y PVY^O. Aquí nosotros identificamos los diferentes tipos de unión en la proteína de la cápside de PVY para tres de los principales cuatro MAbs específicos para PVY^N, Bioerba-N, SASA-N, y Neogen-N, disponibles comercialmente. Se mapearon estos dominios de unión mediante una combinación de prueba de TAS-ELISA de MAbs en múltiples aislamientos de referencia de PVY, análisis de secuencia, expresión heteróloga de fragmentos de la proteína de la cápside, y con experimentos de unión de péptidos sintéticos.

Se encontró que los tres MABs se unen a epítopes lineales localizados dentro de los primeros 31 aminoácidos N-terminales de la proteína de la cápside. El epítipo MAB de Bioreba-N abarcó aa 1-17 e incluyó tres posiciones, aa 1, aa 11, y aa 17, que difieren entre los serotipos PVY^N y PVY^O. Los epítipes SASA-N y Neogen-N abarcaron aa 22-30, e incluyeron dos posiciones, aa 24 y aa29, lo cual difiere entre los serotipos PVY^N y PVY^O. Los epítipes para los MABs SASA-N y Neogen-N es probable que sean idénticos o que se traslapen. Examinando la disponibilidad de secuencias para los aislamientos de PVY de tubérculo necrótico que no reaccionan con MABs específicos para SASA-N y Neogen-N, indicaron una posible selección para sustituciones en epítipes correspondientes que condujeron a la pérdida de reactividad hacia estos anticuerpos. Los datos obtenidos sugieren que probando con mas de un serotipo MAB específico para PVY^N se pudiera asegurar una identificación serológica confiable de aislamiento de PVY^N o PVY^{NTN}.

Keywords PVY · Serotype · Tuber necrosis

Introduction

Potato virus Y (PVY) is a type member of the family *Potyviridae*, and the genus *Potyvirus*. PVY has a positive-stranded RNA genome of ca 9.7-kb with a VPg protein covalently attached to the 5'-terminus and a poly(A) at the 3'-terminus. The PVY genome is expressed as a large single polyprotein later cleared by three virus-specific proteases into 10 mature proteins. The virus has flexuous, filamentous particles comprised of multiple copies of a single capsid protein (CP) species containing 267 amino acids. PVY may be transmitted from plant to plant mechanically, however, in nature the virus is transmitted by aphids in a non-persistent manner.

PVY infects four major agricultural crops, potato, pepper, tomato, and tobacco, and currently represents a major problem for potato industry, especially the seed potato industry (Gray et al. 2010). PVY occurs on all continents, in all major potato producing areas, and thus represents a global problem. The virus affects potato in two different ways—it reduces yield by up to 50%, but can also affect tuber quality inducing potato tuber necrotic ringspot disease (PTNRD) and rendering such tubers completely unmarketable as fresh potato. Most isolates of PVY that induce PTNRD belong to the PVY^{NTN} strain (Beczner et al. 1984; Singh et al. 2008) and are considered regulated pathogens between many international seed and ware potato trading partners (Anonymous 2003; Karasev et al. 2010). Although both serological and RT-PCR molecular typing techniques are available to identify the PVY^{NTN} strain, a majority of regulatory agencies and seed certification programs rely on ELISA.

The overwhelming majority of PVY isolates inducing PTNRD have a PVY^N serotype, and in the U.S. and Canada, most of the isolates having a PVY^N serotype belong to the PVY^{NTN} strain (Gray et al. 2010). Therefore, the PVY^N serotype has been a relatively straightforward and simple serological marker for PTNRD. Consequently, there has been a high demand for specific monoclonal antibodies (MABs) capable of identifying the PVY^N serotype from regulatory agencies and potato industries. At least four commercial MABs are currently in use for screening of tuber necrotic PVY isolates on a large scale in different parts of the world. Serotype specificities of the available MABs vary, and it has long been accepted that these specificities are not 100% strict, assuming some limited cross-reactivity with PVY isolates having other serotypes (Ellis et al. 1996; Karasev et al. 2010). Nevertheless, these commercial MABs have served the potato industry quite well for the past almost 30 years.

Initial attempts to produce PVY^N-specific MABs date back to the early 1980s, when several were generated in Europe and Canada (Gugerli and Fries 1983; Rose and Hubbard 1986; McDonald and Kristjansson 1993; Ellis et al. 1996). Two of these PVY^N-specific MABs, 1F5 (Ellis et al. 1996) and Bioreba-N (Gugerli and Fries 1983) are now marketed commercially by Agdia, Inc. (Elkhart, IN) and Bioreba, AG (Reinach, Switzerland), respectively. Two additional PVY^N-specific MABs, SASA-N (Karasev et al. 2010) and Neogen-N are marketed by Scottish Agricultural Science Agency (SASA, Edinburgh, Scotland) and Neogen Europe, Ltd. (Ayr, Scotland), respectively. All four MABs are used world-wide on a relatively large scale for testing seed and ware potatoes traded on the international and national markets. However, specificities of these MABs and locations of the corresponding epitopes on the PVY antigen have not been systematically studied. MAB 1F5 was shown to have a conformational, SDS-sensitive epitope, with aa 98 residue in the PVY CP involved in the antibody binding (Karasev et al. 2010). On the other hand, MAB SASA-N was found to bind a linear, SDS-insensitive epitope distinct from the 1F5 epitope (Karasev et al. 2010). No other information is currently available on epitope structure, location, and specificity for the main PVY^N serotype-specific MABs.

In the past few years, the number of reported misidentifications of PVY strains based on serological data have been increasing (Chikh Ali et al. 2007; Karasev et al. 2010; Galvino-Costa et al. 2012), and PVY capsid protein evolution and strain selection were hypothesized as a contributing factor (Karasev et al. 2010, 2011; Galvino-Costa et al. 2012), prompting renewed interest in the study of MAB specificity and the mapping of binding domains. To understand the failure of certain PVY^N-specific commercial antibodies to detect some PVY^N or PVY^{NTN} isolates, we conducted a systematic study of the epitope specificities of the three main commercial MABs specific to PVY^N-serotype,

Bioreba-N, SASA-N, and Neogen-N. MAb binding sites were mapped through a combination of TAS-ELISA testing of MAbs on multiple reference isolates of PVY, sequence analysis, heterologous expression of capsid protein fragments, and synthetic peptide binding experiments. Epitope structure and positions were defined for all three MAbs having linear, SDS-insensitive binding sites.

Materials and Methods

Polyclonal and Monoclonal Antibodies

Two polyclonal antisera, rabbit UID8 and goat G500, made against PVY PB-Oz, a common isolate of PVY^O, were described previously (Karasev et al. 2010). Both antisera were able to detect all tested PVY strains including those with the PVY^N serotype, like PVY^N, PVY^{NTN}, PVY^{NA-N}, PVY-NE11, PVY^Z, and PVY^E (Karasev et al. 2010, 2011; Kerlan et al. 2011; Galvino-Costa et al. 2012). Four PVY^N strain-specific monoclonal antibodies were used, 1F5 (Ellis et al. 1996, 1997) obtained from Agdia (Elkhart, IN), SASA-N (Karasev et al. 2010) obtained from SASA, (Edinburgh, Scotland), Neogen-N obtained from Neogen Europe, Ltd. (cat. #1051-02, Glasgow, UK), and Bioreba-N obtained from Bioreba, AG (cat. #112722 and #112712, Reinach, Switzerland). All four monoclonal antibodies, 1F5, SASA-N, Neogen-N, and Bioreba-N are specific to the PVY^N and PVY^{NTN} strains, and do not react with the PVY^O, PVY^{N-Wi} and PVY^C strains.

Virus Isolates

Twenty-three PVY isolates collected from potato in the U.S. between 2001 and 2008 were described previously (Baldauf et al. 2006; Hu et al. 2009b; Lorenzen et al. 2006; Karasev et al. 2011); four isolates collected in Brazil in 2009 were included in the experiments due to their unusual serological properties (Galvino-Costa et al. 2012). Molecular properties and PVY strain group assignments for the PVY isolates used for analysis are summarized in Supplementary Table 1. All isolates were maintained as lyophilized and/or frozen tissue from mechanically inoculated tobacco plants and by mechanical plant-to-plant transfer using tobacco plants maintained in an insect-free, isolated growth chamber.

Peptides

Synthetic peptides were selected based on multiple alignments of the CP sequences for PVY isolates from major strain groups and preliminary ELISA data on specificity of the PVY^N-specific monoclonal antibodies. The peptides used for analysis and their respective sequences are listed in Table 1. Thirteen peptides were synthesized by GenScript

USA, Inc. (Piscataway, NJ) and obtained as lyophilized powder at >85% purity based on HPLC data. Peptides were dissolved in double-distilled water at 10 mg/ml and these stock solutions were stored at -20°C until use.

Bacterial Expression of the PVY CP Gene Fragments and Western Blots

To facilitate the epitope identification, in the cloned and expressed N-terminal CP fragment the first G residue characteristic of N-serotype (Fig. 1) was replaced with the A residue, characteristic of the O-serotype of PVY. Thus, the 5'-terminal fragment of the CP gene for different PVY isolates was amplified from nucleic acid extracts of PVY-infected tobacco leaf tissue samples using a RT-PCR protocol (Hu et al. 2009b) and two specific primers, CPYnF 5'-GGTGAATTCGCAAATGACACAATCGAT-3' and CPYnR 5'-GGTCTGCAGGTATCAAACACTGT-3', the *EcoRI* and *PstI* sites are underlined. Virus-specific sequences were amplified between positions corresponding to nt 8,571–8,846 in the genome of PVY-N4 (GenBank accession number FJ204164). The amplified 275-bp CP gene fragment was cloned into a pMAL-c2 expression vector (New England Biolabs, Ipswich, MA) between *EcoRI* and *PstI* sites. Resulting recombinant plasmid constructs were expressed in *E. coli* after induction with IPTG, according to manufacturer's instructions, and the ca. 50-kDa fusion protein (FP) carrying a maltose-binding fragment (MBP) was denatured with SDS and electrophoretically separated directly from bacterial extracts without purification. These FP bands were probed with various polyclonal and monoclonal antibodies in Western blots. Briefly, leaf samples from tobacco plants infected with PVY isolates or bacterial cells were ground in the Laemmli Tris-SDS sample buffer and heated at 95°C for 4 min. Proteins were separated on 4–20% gradient polyacrylamide gels using Laemmli's Tris-SDS protocol (Bio-Rad, USA) and transferred onto a nitrocellulose membrane (Bio-Rad, USA). 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, washed in PBS containing 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.

ELISA Formats and Competitive Assays

ELISA protocol was based on the previously published methodology (Nikolaeva et al. 1997; Karasev et al. 2010).

Table 1 A list of synthetic peptides used for epitope analysis and summary of the reactivities of the three PVY^N-specific MAbs towards synthetic peptides in a direct (D) or competitive (C) assay

Peptide ID	Peptide length, aa	Sequence ^{a)}	Bioreba-N		SASA-N		Neogen-N	
			D ^{b)}	C ^{c)}	D ^{b)}	C ^{c)}	D ^{b)}	C ^{c)}
1. mon17_31	15	QEQGSIQPNLNKEKE	-	-	+++	++	+++	++
2. ast17_31	15	QEQGIQPNLNKEKE	-	-	+++	++	+++	++
3. aga17_31	15	QEQGSIQPNLIKEKE	-	-	-	-	-	-
4. 423_17_31	15	QEQGSIQPNLSKEKE	-	-	-	-	-	-
5. pvy12_17_31	15	QEQGSIQPNLNKGKE	-	-	-	-	-	-
6. unk17_31	15	QEQGSIQPNLNKEKD	-	-	+++	++	+++	++
7. hr1_1-17	17	GNDTIDAGGSTKKDAKQ	+++	++	-	-	-	-
8. id20_1-17	17	GNDTIDAGGSNKKDAKQ	-	+	-	-	-	-
9. l26_8-27	20	GGSTKKDAKQEQGSIQPNLN	-	-	-	-	-	-
10. 423_8-27	20	GGSTKKDAKQEQGSIQPSLN	-	-	-	-	-	-
11. hr1_1-27	27	GNDTIDAGGSTKKDAKQEQGSIQPNLN	+++	++	-	-	-	-
12. 423_1-27	27	GNDTIDAGGSTKKDAKQEQGSIQPSLN	+++	++	-	-	-	-
13. id20_1-27	27	GNDTIDAGGSNKKDAKQEQGSIQPNLN	+++	+	-	-	-	-

^{a)} Peptide sequences are aligned according to the positions in the PVY CP alignment presented in Fig. 1.
^{b)} Detection of peptides immobilized on polysterene ELISA plates in indirect ELISA format: -, OD₄₀₅ signal is equal to a negative (no peptide) control; +++, OD₄₀₅ signal is more than 3 times higher than for a negative control
^{c)} Detection of isolate NE-11 in tobacco in the presence of peptides listed: -, OD₄₀₅ signal is not reduced (=equal to the control with no peptide added); +, OD₄₀₅ signal is reduced more than 25% to the control with no peptide added; ++, OD₄₀₅ signal is reduced more than 90% to the control with no peptide added; NT = not tested

Briefly, for TAS-ELISA, wells of Nunc MaxiSorp microtiter plates (Nunc, Rochester, NY) were coated with 200 µl of the G500 polyclonal 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

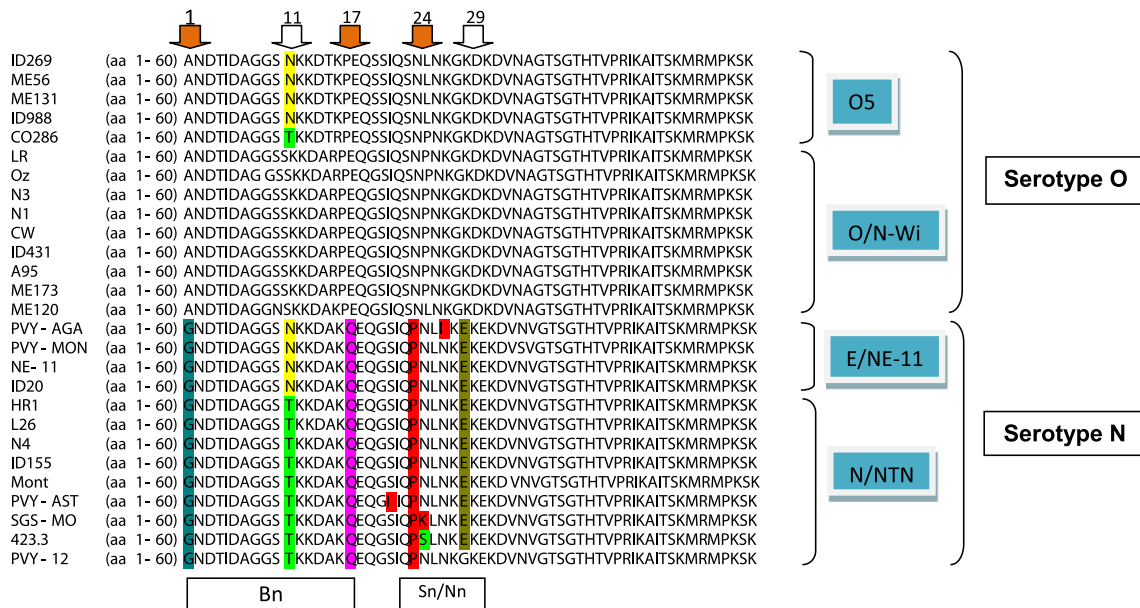


Fig. 1 Multiple alignment of the N-terminal CP sequences (aa 1-60) for different PVY isolates. Brackets to the right designate PVY strain groups and serotypes. Rectangles below the alignment mark epitope positions determined in this work for Bioreba-N (Bn), and for SASA-N and Neogen-N (Sn/Nn). Arrows above the alignment designate amino acid

positions that differ between serotypes O and N, numbers show the amino acid position; solid arrows indicate positions with a clear-cut difference between O and N serotype, and an empty arrows show the positions where the difference between O and N serotype is not clear-cut

times with deionized water, and 200 μ l of plant extract was loaded into each well. To analyze PVY-infected tobacco plants, one-gram samples of green leaf tissue were homogenized in 10 ml of extraction buffer (1 \times phosphate-buffered saline, 0.5% Tween-20, 0.3% dry milk, 2% polyvinylpyrrolidone). Plates were incubated with plant extracts for 16 to 20 h at 4°C, washed with PBST, and an intermediate detecting polyclonal antiserum UID8 or monoclonal antibodies under testing at the appropriate concentration were 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. Goat anti-rabbit (Sigma A-3687) or goat anti-mouse (Sigma A3562) IgG-conjugate with alkaline phosphatase at 1:30,000 dilution in PBST with 0.2% BSA was 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 3 \times the healthy controls.

Two formats were used for peptide reactivity testing: a) indirect ELISA with tested peptides bound directly to the plate; and b) competitive assay to identify if the individual peptides could inhibit the binding of the MAbs to PVY in TAS-ELISA. For the direct plate binding experiments, 200 μ l of the peptide solutions (10–100 μ g/ml) in coating buffer were loaded per well and incubated overnight at 4°C, and then wells were blocked with 400 μ l of 3% non-fat milk in 1 \times PBS, 4–8 h at 4°C. Other steps were the same as described for TAS-ELISA. For the competitive assays, extracts from tobacco plants infected with individual PVY isolates were added to G500 coated wells as described for TAS-ELISA. The individual MAbs were mixed with each peptide (10 μ g/ml) and incubated overnight at 4°C prior to adding to the wells. MAb controls were incubated the same way without peptides added. Other steps were the same as in the TAS-ELISA protocol. Changes in MAb reactivity to specific PVY isolates were then recorded as differences in ELISA signal in the presence or in the absence of individual peptides.

Results

Detection of PVY Isolates by N-Specific Monoclonal Antibodies

Serological reactions of nine PVY isolates with fully-sequenced whole genomes were determined using four commercial MAbs marketed as being specific to the PVY^N

serotype. The isolates included one representative from the PVY^N (Mont) and PVY^Z (L26) strain groups, two from the PVY^E strain group (MON, AGA), four from the PVY^{NTN} strain group (N4, 423.3, AST, SGS-MO), and the unclassified isolate, NE-11 (Lorenzen et al. 2008). Four of these isolates (AST, SGS-MO, MON, AGA) were collected in Brazil and displayed unusual serology and/or recombinant patterns (Galvino-Costa et al. 2012; this work). The remaining five isolates were collected in the U.S. between 2004 and 2010 and were described previously (Lorenzen et al. 2006; Hu et al. 2009b; Karasev et al. 2010). All of the isolates were expected to have a N-serotype, however, three of the commercial antibodies, SASA-N, Neogen-N, and Bioreba-N, were not able to identify all the isolates (Table 2). The SASA-N and Neogen-N antibodies were unable to detect two or three of the isolates from Brazil, while the Bioreba-N antibody did not detect the 423.3 isolate from the U.S. The 1F5 antibody detected all nine isolates. The 1F5 antibody was shown previously to bind a conformational epitope distinct from linear epitope recognized by SASA-N (Karasev et al. 2010).

Heterologous Expression of the N-Terminal Fragment of PVY CP

To determine if the MAbs were binding to the amino terminal end of the CP, a highly antigenic region of most potyviruses (Shukla et al. 1989), a 5'-terminal 275-bp fragment of the CP gene for five of the nine isolates used in the ELISA was cloned and the 92-aa segments were expressed in bacteria. The three MAbs (1F5 was not tested due to its known binding of a conformational epitope) were able to recognize

Table 2 Reactivity of PVY isolates exhibiting the PVY^N serotype against four N-specific MAbs in triple-antibody sandwich (TAS)-ELISA. Tobacco plants infected with the PVY isolates listed were used in ELISA tests for analysis; MAbs were used as detecting antibodies with polyclonal antibodies (PAb) as positive control

Isolate	PAb	SASA-N	Neogen-N	Bioreba-N	1F5
PVY ^N -Mont	+ ^{a)}	+	+	+	+
PVY ^Z -L26	+	+	+	+	+
PVY ^{NTN} -N4	+	+	+	+	+
PVY ^{NTN} -423.3	+	+	+	- ^{b)}	+
PVY ^{NTN} -AST	+	-	-	+	+
PVY ^{NTN} -SGS-MO	+	-	NT ^{c)}	NT	+
PVY-NE11	+	+	+	+	+
PVY ^E -MON	+	+	+	+	+
PVY ^E -AGA	+	-	-	+	+

^{a)} +, OD₄₀₅ signal is more than 3 times higher than for a negative control

^{b)} -, OD₄₀₅ signal is equal to a negative control

^{c)} NT = not tested

the N-terminus of the CP using Western blots and the specificity was similar to that determined using TAS-ELISA (Table 3). SASA-N and Neogen-N antibodies recognized each of the isolates with the exception of AST and AGA. Bioreba-N did not recognize 423.3, but also did not recognize MON, whereas this isolate was recognized in the TAS-ELISA. Since in the cloned and expressed N-terminal CP fragment the first G residue characteristic of N-serotype was replaced with the A residue, characteristic of the O-serotype of PVY (see Fig. 1), the lack of reactivity to the cloned N-terminal fragment of PVY-MON may be interpreted as an indication of a specific role for the aa at position 1 replaced in every cloned construct, including PVY-MON. This aa 1, G in PVY^N serotype, was thus placed in the epitope recognized by Bioreba-N, and designated Bn (Fig. 1).

Based on the data obtained for ELISA tests and for Western-blot analysis of the cloned N-terminal fragments, we concluded that all three MABs recognize epitopes located within the N-terminal 92 amino acid residues of the PVY CP. The fact that Bioreba-N, SASA-N, and Neogen-N MABs were able to recognize PVY CP and its N-terminal fragment in Western blots, further suggested that these epitopes belonged to the SDS-insensitive, linear, probably continuous type.

CP Sequence Alignments and MAB Reactivities to Synthetic Peptides

When the PVY CP sequences for the isolates screened by TAS-ELISA were aligned, using the ClustalX program, with other selected PVY CP sequences, the majority of variable amino acid positions were clustered near the N-terminus of the CP (Fig. 1). One specific variable position located in the middle of the CP, at aa 98, was previously correlated to the reactivity with another N-specific MAB, 1F5, and was not addressed in this work due to the conformational nature of the 1F5 epitope (Karasev et al. 2010). At least seven

Table 3 Serological reactivity of the bacterially expressed N-terminal 92-aa capsid protein (CP) fragments in Western blots. CP fragments were cloned and expressed as ca. 50-kDa fusion proteins for individual listed PVY isolates, and reactivity towards the three PVY^N-specific MABs was tested for each, with polyclonal antibodies (PAb) as positive control

Isolate	PAb	SASA-N	Neogen-N	Bioreba-N
PVY ^N -Mont ^{a)}	+	+	+	+
PVY ^{NTN} -N4	+	+	+	+
PVY ^{NTN} -423.3	+	+	+	–
PVY ^{NTN} -AST	+	–	–	+
PVY ^E -MON	+	+	+	–
PVY ^E -AGA	+	–	–	+

^{a)} Full-size, 267-aa CP

variable positions could be identified within the first 36 N-terminal amino acids of the PVY CP with some degree of correlation to a serotype of a corresponding isolate (Fig. 1). The two isolates from the NE-11 group (NE-11 and ID20) had substitutions at aa 11, the 423.3 isolate had a substitution at aa 25, while the four Brazilian isolates, PVY-MON, PVY-AGA, PVY-AST, and SGS-MO, had additional substitutions between aa 19–25, that helped to focus our attention in on the variable positions at aa 11, 17, and 29.

A series of 13 peptides was synthesized spanning the 31 N-terminal amino acids, ranging in length from 15 to 27 aa (Table 1). All 13 were easily soluble in water and were subjected to two types of assays. In a direct assay, the peptides were immobilized onto a polystyrene surface of the ELISA plate, and their reactivity towards the three N-specific MABs was assessed in ELISA as outlined in **Materials and Methods**. In this format, peptide reactivity was tested when bound to the solid support. In a competitive assay, peptides were pre-incubated with MABs under testing prior to loading these MABs as detecting antibodies in TAS-ELISA. In this format, peptide reactivity was tested while in solution, providing a different environment for antibody binding as compared to the solid support binding. Both formats provided consistent data, although the competitive format demonstrated slightly broader specificity for one peptide; these data are summarized in Table 1. Apparently, all three MABs react to the N-terminal fragment of the CP in Western blots (Table 3), and all three bind peptides from the first 31 aa region of the CP (Table 1). Consequently, it can be concluded that the three MABs recognize two distinct epitopes located within the first 30 aa of the CP.

Bioreba-N MAB binds peptides spanning the aa 1–17 region, and thus likely recognizes an epitope located within the first 17 N-terminal amino acid residues. This epitope includes three variable positions at aa 1, 11, and 17 displaying polymorphism between isolates with PVY^N and PVY^O serotypes (Fig. 1). Interestingly, two variable positions, aa 1 and aa 11, seem to allow certain flexibility for the reactivity to Bioreba-N: substitutions of the threonine residue for the glutamate in PVY^E/NE-11 sequences at position 11 (Fig. 1) do not immediately lead to the loss of Bioreba-N reactivity—only when an additional position, aa 1, is changed to an O-specific alanine residue, the reactivity is lost for PVY-MON (Table 3).

SASA-N and Neogen-N bind peptides spanning aa 22–30; this epitope is quite distinct from the Bioreba-N epitope, and includes two variable positions, aa 24 and aa 29 which differ between PVY isolates with PVY^N and PVY^O serotypes (Fig. 1). Three tested substitutions in this region, at aa 25 (N/K), aa 27 (N/I or N/S), and aa 29 (E/G), resulted in complete loss of the reactivity to both SASA-N and Neogen-N MABs, while one substitution at aa 25 (N/S) characteristic of the PVY^{NTN} isolate 423.3 did not affect

SASA-N or Neogen-N binding (Fig. 1; Tables 2 and 3). Interestingly, a substitution at aa 21 (S/I) did not affect binding of peptide ast17_31 to either of the MAbs (Table 1), although the corresponding PVY isolate PVY-AST displayed a complete loss of reactivity to both SASA-N and Neogen-N MAbs in TAS-ELISA and in Western blots (Tables 2 and 3). Because of this, we excluded the aa 21 position from the identified epitope for SASA-N and Neogen-N MAbs. This position, aa 21, is likely outside of the “core” epitope recognized by these two MAbs, but apparently can affect the binding when placed in a longer polypeptide chain. However, aa 31 position, which is a variable, polymorphic position differing between PVY^N and PVY^O serotypes, is clearly located outside of the Sn/Nn epitope, since the peptide “unk17_31” with a substitution at aa 31 (E/D) binds both MAbs in a direct plate assay, and fails to block binding with the virus antigen in a competitive assay (Table 1).

Discussion

The PVY genome is documented to have a tremendous diversity due to recombination and mutations, providing huge pool of variants necessary for PVY to survive and succeed in different hosts and environments (Kerlan 2006; Blanchard et al., 2008; Hu et al. 2009a; Gray et al. 2010). Given such a diversity and plasticity of the PVY genome, regulation of the potato trade and efforts to limit spread of the most damaging tuber-necrotic PVY isolates have recently become a challenge (Karasev et al. 2010). Specifically, a recent identification of two tuber-necrotic PVY isolates from Brazil, PVY-AST and PVY-AGA that failed to react to the SASA-N monoclonal antibody (Galvino-Costa et al. 2012) highlighted an apparent lack of understanding of epitope specificities for commercial PVY^N-serotype specific MAbs.

In this work, two distinct epitopes have been identified on the PVY CP, one (Bn, aa 1-17) binding Bioreba-N, and another (Sn/Nn, aa 22-30) binding both SASA-N and Neogen-N antibodies. Both epitopes recognized by these three PVY^N-serotype specific antibodies, belong to the SDS-insensitive, linear type, and retain their structure and reactivity after denaturation and solid support immobilization. Additionally, regions outside the 17 amino acid Bn epitope and the 9 amino acid Sn/Nn epitope can affect antibody binding. For example, a single N/S substitution at position 25 in isolate 423.3, outside the Bn epitope, abolishes Bioreba-N binding, detectable both in TAS-ELISA and Western blots on plant extracts or bacterially produced proteins, but not when synthetic peptides are tested (Tables 1, 2, and 3). Another substitution at position 27 in isolate PVY-AGA, again outside of the Bn epitope, also affects reactivity of the Bioreba-N antibody, apparently restoring the reactivity of the cloned N-terminal fragment of the CP with aa

1 replaced with a PVY^O-specific residue (Tables 2 and 3). Similarly, a single S/I substitution at position 21 in isolate PVY-AST, outside of the Sn/Nn epitope, leads to a complete loss of reactivity with SASA-N and Neogen-N, but detectable only in TAS-ELISA (Tables 1, 2, and 3).

Examination of the CP sequences for the three Brazilian PVY isolates, PVY-AGA, PVY-AST, and SGS-MO, that failed to bind SASA-N antibody but did bind another PVY^N-serotype specific antibody 1F5 (Galvino-Costa et al. 2012; this work), suggests that this failure occurred due to a series of mutations in or close to the Sn/Nn epitope, positions 21, 25, and 27 (Fig. 1; Table 1). It is remarkable that these three PVY isolates from Brazil collected during the same season of 2008, from two different states of Brazil exhibit such a tight clustering of three independent mutations affecting the same Sn/Nn epitope (Fig. 1). This clustering may indicate that these SASA-N/Neogen-N-negative PVY variants were selected for by relying only on the use of one or both of these MAbs to detect and remove potatoes infected by the PVY^N serotype. One other mutation, substitution E/G at position 29, characteristic of the tuber-necrotic isolate PVY-12 (Chikh Ali et al. 2007) would also affect the Sn/Nn epitope leading to the loss of reactivity with both SASA-N and Neogen-N MAbs in a direct peptide binding format (Table 1). Although neither SASA-N or Neogen-N were tested in ELISA against PVY-12 in the original publication (Chikh Ali et al. 2007), we can hypothesize that both would have been negative based on the Sn/Nn epitope position and peptide reactivity data (Table 1).

The data obtained in this work and data published elsewhere (cf. Chikh Ali et al. 2007; Karasev et al. 2010; Galvino-Costa et al. 2012) allowed us to identify at least three distinct, non-overlapping epitopes specific for the PVY^N serotype of PVY. Two epitopes, Bn and Sn/Nn, are linear, SDS-insensitive, located close to the N-terminus of the CP. One epitope, for the 1F5 MAb, is conformational; location for this epitope is not yet mapped precisely, although aa 98 was found involved in this epitope (Karasev et al. 2010). It is important to note that the two commercial MAbs, SASA-N and Neogen-N, have either identical or almost completely overlapping epitopes—herewith designated as a single epitope Sn/Nn (Fig. 1).

With these epitope mapping data available, can a PVY^N-serotype for PVY be defined? This question has very important practical implications, since all the commercial PVY^N-specific MAbs are extensively used in regulating the potato trade, both ware and seed. Based on the reactivities of the PVY isolates studied to date we can provide the following working definition of a PVY^N-serotype. We believe that to be considered a PVY^N type serologically, a PVY isolate must react positively to at least one N-specific MAb (1F5, SASA-N, Neogen-N, Bioreba-N), and react negatively to PVY^O-specific MAbs, but testing with multiple PVY^N-specific MAbs will greatly increase the confidence of correctly

identifying isolates belonging to the PVY^N-serotype. For instance, if a point mutation disrupts an epitope recognized by a PVY^N-specific MAb, like Sn/Nn epitope in PVY-12 isolate (Chikh Ali et al. 2007), the other two PVY^N-specific epitopes, Bn and 1F5, would still be intact and provide the correct identification of this isolate as belonging to the PVY^N-serotype. Thus, if relying only on serological identification of PTNRD-inducing isolates of PVY, multiple MAbs specific to the PVY^N-serotype should be used, along with a PVY^O-specific MAb. But it must be pointed out that SASA-N and Neogen-N have identical or very similar epitopes and consequently should be treated as antibodies with identical specificities.

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