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Characteristics of a resistance-breaking isolate of potato virus Y causing potato tuber necrotic ringspot disease

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Abstract. An Austrian isolate of potato virus Y^{NTN}, the causal agent of potato tuber necrotic ringspot disease (PTNRD), was serologically compared with seven Dutch PVY^N isolates. Using polyclonal and monoclonal antibodies, it was found indistinguishable from PVY^N. Determination of the nucleotide sequence of the coat protein cistron and comparison of the deduced amino acid sequence with coat protein sequences of other potyviruses revealed a high level of homology with PVY^N coat protein sequences. This confirmed the close taxonomic relationship of PVY^{NTN} with the PVY^N subgroup of potato virus Y. PVY^{NTN} is able to overcome all resistance genes known so far in commercial potato cultivars. Remarkably, transgenic PVY-protected tobacco plants are also resistant to PVY^{NTN} infection upon mechanical and aphid-mediated inoculation. These experiments indicate that genetically engineered resistance offers great potential in protection of potato to new aggressive strains of PVY^N.

Introduction

Etiological studies have indicated that the potato tuber necrotic ringspot disease (PTNRD) is associated with the presence of potato virus Y (PVY) isolates [Weidemann, 1985], particularly the tobacco veinal necrosis strain (PVY^N) [Beczner et al., 1984; Le Romancer and Kerlan, 1991; Schiessendoppler, 1990, 1992]. At present this disease is a major concern to potato growers and breeders. Not only because of the necrotic ringspot symptoms it causes on potato tubers and concomitant yield losses, but particularly since it affects cultivars which are either immune [Le Romancer and Kerlan, 1992] or possess high levels of field resistance to PVY [Beczner et al., 1984; Steinbach and Hamann, 1989; Buturovic and Kus, 1989; Le Romancer and Kerlan, 1991].

The causal agent of PTNRD is readily detected by polyclonal antibodies to PVY and by monoclonal antibodies specific for PVY^N and is currently referred to as PVY^{NTN} [Le Romancer and Kerlan, 1991; Le Romancer et al.,

1994]. However, the symptoms it causes on *Chenopodium amaranticolor* and on primarily infected potato plants closely resemble those commonly associated with PVY^C or PVY^O infections [Le Romancer and Kerlan, 1991]. Also, an infection with PVY^N does not have an antagonistic effect on PTNRD in cross protection tests [Beczner et al., 1984, Le Romancer et al., 1994].

PTNRD has been reported from Hungary [Beczner et al., 1984], former Yugoslavia [Buturovic and Kus, 1989], Germany [Weidemann, 1985; Steinbach and Hamann, 1989], Denmark [Nielsen, 1992], Austria [Schiessendoppler, 1990], France, Belgium and Lebanon [Le Romancer and Kerlan, 1991]. As the disease has already reached an epidemic state in some of these countries [Kus, 1990, 1992], specific detection of the virus and identification of sources of resistance gene(s) are of extreme importance to the various seed potato programmes.

In this paper we present data on the immunological detection of an Austrian isolate of PVY^{NTN} reported to induce PTRND [Schiessendoppler, 1990, 1992], using poly- and monoclonal antibodies (MAbs) to PVY^N. The nucleotide sequence of the coat protein (CP) cistron was determined and its deduced amino acid sequence compared to other potyviruses and PVY isolates. In addition, transgenic tobacco plants transformed with the PVY^N CP gene and previously shown to be protected against mechanical and aphid-inoculation with PVY^N and closely related viruses [Van der Vlugt et al., 1992, Van der Vlugt and Goldbach, 1993], were tested for their resistance toward PTNRD.

Materials and methods

Virus isolates, antisera and aphids

Tubers of the potato cultivar Hermes infected with the causal agent of PTNRD were kindly provided by Dipl. Ing. E. Schiessendoppler (Bundesanstalt für Pflanzenschutz, Wien) and maintained under quarantine conditions in compliance with Dutch regulations. Seven PVY^N isolates, i.e. #601–#607 were derived from the IPO-DLO virus collection. Polyclonal antibodies and five MAbs, raised to PVY^N isolate #603 [Boonekamp and Pomp, 1985] were generously supplied by Ing. D. Z. Maat (IPO-DLO) and Mr. H. Pomp (Laboratory for Monoclonal Antibodies, Wageningen), respectively. The MAbs, designated 4H11A2, 5B8G10, 5C12F9, 8H6A11 and B5C11, were purified from mouse ascitic fluids by ammonium sulphate precipitation [Clark and Adams, 1977]. The polyclonal antibodies were conjugated to alkaline phosphatase using glutaraldehyde [Avrameas, 1969].

Five-day-old *Myzus persicae* (Sulz.) nymphs, biotype WMp2, were reared on *Brassica napus* L. subsp. *oleifera* (oilseed rape) at 20 ± 3 °C and a photoperiod of 16 h per day as described by Van den Heuvel and Peters [1989].

Transgenic plants and resistance screening
PVY^N CP^{+ATG} transgenic *Nicotiana tabacum* var. SR1 lines A30, A809, PVY^N CP^{-ATG} tobacco line D25, and transformation vector pGH2 transformed control plants SR1 (henceforth on referred to as SR1) [Van der Vlugt et al., 1992], were maintained at 20 °C ± 2 and a photoperiod of 16 h per day. S1 progeny plants of self-pollinated original transformants were either aphid- or mechanically inoculated six weeks after sowing (four-leaf-stage). For mechanical inoculation, the two largest leaves were dusted with carborundum powder and extracts of virus-containing leaf material, diluted 20-fold in 0.03 M phosphate buffer, pH 7.7, were used as inoculum. Aphids were given a 10 minutes acquisition access period (AAP) on virus-infected potato plants and then transferred to the tobacco lines for an overnight inoculation access period (IAP). Twenty aphids were used per plant. Forty plants of each tobacco line, arranged in four blocks of ten plants, were tested per treatment. Mock-inoculated controls using aphids directly taken from oilseed rape plants were incorporated. All experiments were carried out under safe greenhouse conditions (PKII-level) as imposed by the national authorities.

ELISA
The presence of viral antigen was monitored in either a cocktail-ELISA [Van den Heuvel and Peters, 1989] using the polyclonal antibodies and conjugate, or in a triple antibody sandwich (TAS)-ELISA when the MAbs were applied, essentially following Van den Heuvel et al. [1990]. The polyclonal antibodies to PVY could easily be used in the serological tests since coat protein production in the transgenic plants was at undetectable low levels [Van der Vlugt et al., 1992]. Preliminary tests showed that ELISA readings at 405 nm (A_{405}) of uninfected transgenic plants never exceeded those of the control line SR1 or wild type tobacco. Controls of uninfected tobacco plants of the various lines were incorporated in ELISA.

Nucleotide sequence determination
Viral RNA was extracted from virus particles purified from *Nicotiana tabacum* cv. White Burley as described by Van der Vlugt et al. [1989]. An 800 basepair (bp) long cDNA fragment, corresponding with the viral CP cistron was generated using RT-PCR (The Gene Amp kit; Perkin Elmer Cetus, Norwalk, CT) and synthetic oligonucleotides 5′-TTTCCATGGG/CAAATGACAATT/CGA-3′ and 5′-TTTCCATGGTCACATGTTCTT/CACTCCAA-3′. The amplified cDNA fragment was digested with NcoI, purified from a 1 % 'low melting point' agarose gel and cloned into the NcoI restriction site of pGEM5Zf(+) (Promega, Madison WI). The nucleotide sequences of cloned inserts were determined using the dideoxy chain termination method [Sanger et al., 1977] with T7 DNA polymerase (Pharmacia LKB Biotech, Uppsala, Sweden) on double-stranded DNA templates [Zhang et al., 1988]. Sequences were aligned and phylogenetic

relationships analyzed using previously described software packages [Van der Vlugt et al., 1993].

Results

Symptomatology
Tubers of potato cultivar Hermes secondarily infected with PTNRD showed symptoms suggestive of potato mop-top virus, tobacco rattle virus (TRV) or PVY^C i.e. superficial necrotic rings and arches, surface blotches, cracks of different sizes and malformation. Secondarily-infected plants initially displayed a clear mosaic on the leaves, and gradually a severe necrosis developed on the stems and veins which eventually resulted in leaf-drop [Beemster and Rozendaal, 1972]. To exclude the involvement of soil-borne viruses in PTNRD, the virus was transmitted by *M. persicae* nymphs to *N. tabacum* cv. White Burley. Furthermore, infected tobacco plants tested negative in ELISA for the presence of the potato viruses S, M, A and X, and potato leafroll virus. PTNRD was then maintained by repeated aphid transfer on tobacco cv. White Burley on which it caused vein clearing, mosaic, and stem and veinal necrosis typical for PVY^N [Beemster and Rozendaal, 1972]. When extracts of these tobacco leaves were used for mechanical inoculation of potato cvs Nicola and Bintje, the tuber progeny gave identical symptoms as observed before on those of cv. Hermes, though at a lower rate for cv. Bintje. In a parallel test, none of the PVY^N isolates used in this study caused tuber symptoms on mechanically inoculated potato cv. Nicola.

Serology
Initial tests with the antiserum raised to PVY^N isolate #603 showed that polyclonal antibodies readily detected PVY^{NTN} in DAS-ELISA but could not be used to differentiate it from other PVY^N isolates. Five MAbs raised to PVY^N isolate #603 were therefore tested on their discriminatory capacity. To this end homogenized tobacco leaf material infected with seven PVY^N isolates and PVY^{NTN} was tested in duplicate in TAS-ELISA using the polyclonal antibodies to sensitize the plates. The mean absorbance value at 405 nm of all PVY^N isolates obtained with a particular MAb was then compared with that of PVY^{NTN} in the same assay (Table 1). All of the MAbs used detected PVY^{NTN} as readily as the PVY^N isolates and no significant differences ($P > 5\%$) were observed (Table 1).

Nucleotide sequence determination and comparison
A number of recombinant plasmids containing PVY^{NTN} CP-specific cDNA inserts were obtained and three of these clones were used to determine the sequence of the PVY^{NTN} CP. Within these three sequences only a few silent point mutations were observed indicating the integrity of the PVY^{NTN} virus

Table 1. Immunological comparison of PVY^N isolates and PVY^{NTN} using five monoclonal antibodies raised against PVY^N #603 coat protein

PVY^N isolates[1]		Monoclonal antibody[2]				
No.	Potato cv. (year of isolation)	4H11A2	5B8G10	5C12F9	8H6A11	B5C11
#601	Alpha (1963)	840[3]	1364	1057	691	1207
#602	Bintje (1978)	584	585	824	410	546
#603	Bintje (1980)	617	643	720	464	588
#604	Bona (1961)	632	1088	1015	654	981
#605	Gineke (1958)	504	589	594	218	523
#606	Hertha (1978)	615	1295	1108	603	1226
#607	Record (1957)	497	781	637	420	698
Mean of PVY^N isolates		613	906	851	494	824
(SE of the mean)		(47)	(138)	(86)	(68)	(126)
95%-confidence limits		512–724	580–1232	648–1054	333–655	526–1122
PVY^{NTN} Hermes (1992)		517	1042	832	391	912

[1] All isolates were transferred to *N. tabacum* 'White Burley' and homogenized leaf materal, diluted 20-fold was tested in duplo.

[2] MAb 4H11A2 was applied at a 64,000-fold, 8H6A11 at a 6,000-fold, and the other MAbs at a 2,000-fold dilution.

[3] The mean absorbance values at 405 nm are presented (× 1,000).

isolate. The nucleotide sequence of the PTNRD coat protein cistron and its derived amino acid sequence are presented in Fig. 1. Comparison of the 267 amino acid long CP sequence with 28 geographically distinct PVY^N and PVY^O isolates revealed a high level of sequence homology both in the complete CP (88.7 to 99.6%) and in the N-terminal region (96.7%). This confirms the close serological relationship to PVY previously observed in the ELISA experiments. Alignment of the PVY^{NTN} CP amino acid sequence with the other PVY CP sequences clearly grouped PVY^{NTN} within the PVY^N subgroup with which it shares a glycine residue as the first CP amino acid as well as a number of non-conserved amino acid mutations in the N-terminus and core region [Van der Vlugt et al., 1993]. This grouping in the PVY^N subgroup was confirmed in a phylogenetic tree generated by the Neighbor program from the PHYLIP programs package [Felsenstein, 1991] (Fig. 2).

Screening for resistance

PTNRD has been reported to break the resistance of a considerable number of potato cultivars with immunity or with high levels of field resistance to PVY^N [Beczner et al., 1984; Steinbach and Hamann, 1989; Buturovic and Kus, 1989; Le Romancer and Kerlan, 1991, 1992]. In a previous report, Van der Vlugt and Goldbach [1993] demonstrated that transgenic tobacco lines transformed with the CP cistron of PVY^N acquired high levels of resistance (up to 95%) against PVY infections, irrespective of the method

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1     GGAAATGACACAATTGATGCAGGAGGAAGCACTAAGAAGGATGCAAAACAAGAGCAAGGT
        G  N  D  T  I  D  A  G  G  S  T  K  K  D  A  K  Q  E  Q  G

61    AGCATTCAACCAAATCTCAACAAGGAAAAGGAAAAGGACGTGAATGTTGGAACATCTGGA
        S  I  Q  P  N  L  N  K  E  K  E  K  D  V  N  V  G  T  S  G

121   ACTCATACTGTGCCACGAATTAAAGCTATCACGTCCAAAATGAGAATGCCCAAGAGTAAA
        T  H  T  V  P  R  I  K  A  I  T  S  K  M  R  M  P  K  S  K

181   GGTGCAACTGTACTAAATTTGGAACACTTACTCGAGTATGCTCCACAGCAAATTGACATC
        G  A  T  V  L  N  L  E  H  L  L  E  Y  A  P  Q  Q  I  D  I

241   TCAAATACTCGAGCAACTCAATCACAGTTTGATACGTGGTATGAAGCGGTACAACTTGCA
        S  N  T  R  A  T  Q  S  Q  F  D  T  W  Y  E  A  V  Q  L  A

301   TACGACATAGGAGAAACTGAAATGCCAACTGTGATGAATGGGCTTATGGTTTGGTGCATT
        Y  D  I  G  E  T  E  M  P  T  V  M  N  G  L  M  V  W  C  I

361   GAAAATGGAACCTCGCCAAACATCAACGGAGTTTGGGTTATGATGGATGGAGATGAACAA
        E  N  G  T  S  P  N  I  N  G  V  W  V  M  M  D  G  D  E  Q

421   GTCGAATACCCACTGAAACCAATCGTTGAGAATGCAAAACCAACCCTTAGGCAAATCATG
        V  E  Y  P  L  K  P  I  V  E  N  A  K  P  T  L  R  Q  I  M

481   GCACATTTCTCAGATGTTGCAGAAGCGTATATAGAAATGCGCAACAAAAAGGAACCATAT
        A  H  F  S  D  V  A  E  A  Y  I  E  M  R  N  K  K  E  P  Y

541   ATGCCACGATATGGTTTAGTTCGTAATCTGCGCGATGGAAGTTTGGCTCGCTATGCTTTT
        M  P  R  Y  G  L  V  R  N  L  R  D  G  S  L  A  R  Y  A  F

601   GACTTTTATGAGGTCACATCACGAACACCAGTGAGGGCTAGGGAAGCGCACATTCAAATG
        D  F  Y  E  V  T  S  R  T  P  V  R  A  R  E  A  H  I  Q  M

661   AAGGCCGCAGCATTGAAATCAGCCCAACCTCGACTTTTCGGGTTGGACGGTGGCATCAGT
        K  A  A  A  L  K  S  A  Q  P  R  L  F  G  L  D  G  G  I  S

721   ACACAAGAGGAGAACACAGAGAGGCACACCACCGAGGATGTCTCTCCAAGTATGCATACT
        T  Q  E  E  N  T  E  R  H  T  T  E  D  V  S  P  S  M  H  T

781   CTACTTGGAGTCAAGAACATG
        L  L  G  V  K  N  M
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of an Austrian isolate of PVY^{NTN}. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X79305.

of inoculation. To test whether this genetically engineered resistance, which was shown to be RNA-mediated, is also effective against PVY^{NTN}, tobacco plants of three resistant transgenic lines (A30 and A809 (CP^{+ATG}) and D25 (CP^{-ATG})) were inoculated with PVY^{N} or PVY^{NTN} either mechanically or by viruliferous aphids. Establishment of virus infection was tested in DAS-ELISA four weeks after inoculation. To this end an inoculated leaf of each plant was sampled twice and a plant was considered infected if the ELISA reading of a leaf sample was higher than the mean value of the negative (mock inoculated) controls plus three times their standard deviation. All transgenic tobacco lines displayed high to significant levels

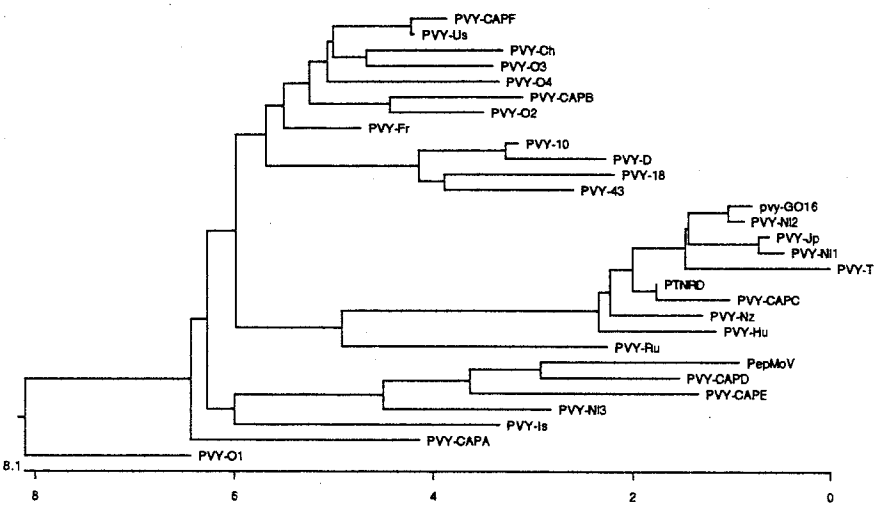

Fig. 2. Unrooted Clustal phylogenetic tree based on multiple alignments of 28 PVY isolates and PVY^{NTN} (PTNRD). For sources of virus isolates see Van der Vlugt et al., 1993. For PVY isolates CAPA – CAPF see EMBL/GenBank accession numbers X68221, X68222, X68223, X68224, X68225 and X68226.

of resistance to infection upon mechanical inoculation with either PVY isolate (Table 2). However, only tobacco lines D25 and A30 were immune to vector-mediated inoculation while remarkably the protection in line A809 failed in this assay. This suggests a fundamental difference in the resistance mechanism in line A809 in comparison to lines A30 and D25. The reduction of vector-mediated infection in the lines D25 and A30 was about 85% for PVY^{N} and more than 90% for PVY^{NTN}. Analysis of S2 progeny lines obtained from self-pollinated protected S1 plants from lines A30 and D25 revealed that the level of resistance to mechanically and vector-mediated infection can be raised to 100% within one generation (results not shown).

Discussion

Serological studies described in this paper have shown that PTNRD is readily detectable by poly- and monoclonal antibodies directed against PVY^{N}. In fact, a panel of five different MAbs raised against PVY^{N} coat protein could not be used to discriminate the agent causing PTNRD from other PVY^{N} isolates although this panel clearly distinguished PVY^{N} from PVY^{O} and PVY^{C} and other unrelated potyviruses [H. Pomp, pers. comm.]. The close serological relationship to PVY^{N} is supported by the nucleotide

and amino acid sequence data of the coat protein gene of PVY^{NTN} (Fig. 1) and its grouping in the PVY^{N} subgroup (Fig. 2). The CP sequence data as compiled from three independently obtained cDNA clones also shows that the Austrian PVY^{NTN} is not contaminated with other PVY or potyvirus isolates. In addition the necrotic ringspots were also observed on tubers of potato cv. Bintje upon mechanical inoculation with the purified virus used for RNA-isolation, indicating that PVY^{NTN} is the causal agent of PTNRD.

Interestingly the coat protein sequence of the Austrian PTNRD isolate shows a high level of homology (98.4%) with a Hungarian PVY isolate which is reported to cause necrotic rings around the buds on tubers and mottling of leaves [Thole et al., 1993]. Though no additional biological or serological data on this isolate is available, the tuber symptoms appear to be characteristic of PVY^{NTN}. A comparison of the complete nucleotide and amino acid sequence of this Hungarian PVY isolate [Thole et al., 1993] with the complete sequence of PVY^{N} [Robaglia et al., 1989], revealed a high level of overall homology and only a limited number of sequence differences. Further sequence analyses of other PVY^{N} and PVY^{NTN} isolates are however needed to establish whether these differences are characteristic of these two isolates. If so, this would enable the development of a PCR-based test employing type-specific primers to distinguish PVY^{N} and PVY^{NTN} virus isolates.

The results summarized in Table 2 clearly show that the resistance in PVY^{N} CP transgenic tobacco lines, which had previously been shown to protect against PVY^{N} and another PVY strain, is also effective against PVY^{NTN}. Two of the three lines tested resisted both mechanical and vector-mediated inoculation of PVY^{N} and PVY^{NTN}. In transgenic line A809 however, viruliferous aphids were readily able to overcome the transgene resistance. An analogous situation was observed in transgenic tobacco plants expressing the CP of TRV, where viruliferous trichodorid nematodes broke the engineered resistance to this virus [Ploeg et al., 1993]. These

Table 2. Percentage* of virus resistance in S1 progeny plants of PVY^{N} CP^{+ATG} transgenic tobacco lines A30, A809, PVY^{N} CP^{-ATG} line D25, and transformation vector pGH2 transformed control line SR1, challenged with PVY^{N} and PVY^{NTN}

Transgenic line	Mechanical inoculation		Vector inoculation	
	PVY^{N}	PVY^{NTN}	PVY^{N}	PVY^{NTN}
SR1	0	0	10 ± 5	34 ± 10
D25	68 ± 10	70 ± 7	85 ± 6	95 ± 3
A30	78 ± 7	90 ± 0	88 ± 3	96 ± 3
A809	60 ± 11	35 ± 17	15 ± 14	40 ± 15

* Percentages ± SE are calculated from four blocks of ten plants each.

results demonstrate that the use of the natural vector should form an essential component in the assessment of resistance to viruses.

The successful identification of resistance to the causal agent of PTNRD in tobacco lines transformed with the CP cistron of PVY^N points to the need to generate PVY^N coat protein cistron transformed potato plants since this form of resistance offers a promising prospect for controlling this important potato disease.

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