

SHORT COMMUNICATION

CHARACTERIZATION OF *BET NECROTIC YELLOW VEIN VIRUS* INFECTING SUGAR BEET IN LITHUANIAM. Zizyte¹, J. Valkonen² and J. Staniulis¹¹ Institute of Botany Nature Research Centre, Zaliuju Ezeru 49, LT-08406, Vilnius, Lithuania² Department of Agricultural Sciences, PO Box 27, 00014 University of Helsinki, Finland

SUMMARY

Beet necrotic yellow vein virus (BNYVV) was shown to occur in sugar beets in three of six regions surveyed in Lithuania. Characterizing the coat protein (CP) and p25 genes by RT-PCR and sequencing showed the presence of A and B types of BNYVV isolates. The p25 protein of Lithuanian BNYVV isolates contained a distinct amino acid tetrad (residues 67-70) with AYHR associated with a B type isolate and AHHG with two A type isolates. These sequence features were consistent with phylogenetic grouping of isolates based on CP and p25 gene sequences. This is the first characterization of BNYVV isolates in Lithuania.

Key words: BNYVV, rhizomania, sugar beet, phylogenetic analysis.

Rhizomania, caused by *Beet necrotic yellow vein virus* (BNYVV, genus *Benyvirus*), was first described in Italy (Canova, 1959) and has spread in most sugar beet-growing areas of the world within a few decades (McGrann *et al.*, 2009; Chiba *et al.*, 2011). BNYVV has a multipartite single-stranded RNA genome with all natural isolates containing four RNA species, although some isolates have an additional RNA-5 (Tamada *et al.*, 1989). RNA-1 and RNA-2 encode proteins involved in viral replication, encapsidation, transmission by *Polymyxa betae* and cell-to-cell movement (Richards and Tamada, 1992; Peltier *et al.*, 2008). RNA-3 and RNA-5 encode pathogenicity factors responsible for rhizomania symptoms expression and root proliferation (Jupin *et al.*, 1992; Link *et al.*, 2005; Klein *et al.*, 2007; Peltier *et al.*, 2011). RNA-4 encodes a 31-kDa protein enhancing vector transmission, symptom severity and RNA silencing suppression in roots (Rahim *et al.*, 2007; D'Alonzo *et al.*, 2012).

On the basis of restriction fragment length polymorphism, single strand conformation polymorphism or sequence analysis of parts of the viral genome, three major serologically indistinguishable BNYVV groups named type A, B and P have been identified. (Kruse *et al.*, 1994; Koenig *et al.*, 1995; Miyanishi *et al.*, 1999; Koenig and Lennefors, 2000). BNYVV A type isolates are distributed worldwide (many European countries, Iran, North America, China and Japan), whereas the B type isolate is prevalent in limited areas of Europe, mainly in France and Germany, but occurs also in China and Japan (Miyanishi *et al.*, 1999; Sohi and Maleki, 2004; Schirmer *et al.*, 2005; Li *et al.*, 2008). Both BNYVV types are distributed in the UK, Sweden Belgium and Iran (Koenig *et al.*, 1995; Lennefors *et al.*, 2000; Sohi and Maleki, 2004; Meunier *et al.*, 2005). BNYVV A and B types typically contain only RNAs 1 to 4. BNYVV P type isolates that contain RNA-5 are detected only in France, Kazakhstan and in the UK (Koenig *et al.*, 1997; Koenig and Lennefors, 2000; Harju *et al.*, 2002; Ward *et al.*, 2007). P type isolates appear to be more virulent than both the A and B types (Heijbroek *et al.*, 1999) and may evade activation of plant defense responses. Therefore P type BNYVV is a considerable potential threat to sugar beet cultivation worldwide where the control of rhizomania is based solely on the use of virus-resistant cultivars (Klein *et al.*, 2007; Peltier *et al.*, 2008). In east Asia RNA-5-containing BNYVV isolates have frequently been found associated with A or B types (Miyanishi *et al.*, 1999; Li *et al.*, 2008). Recently, isolates with the P type p25 gene but lacking RNA-5 have been reported from the USA and Iran (Liu and Lewellen, 2007; Mehrvar *et al.*, 2009). It is believed that A and B type BNYVV became separated a long time ago from the P type-like ancestral isolate, which was probably endemic in east Asia long before sugar beet was cultivated (Chiba *et al.*, 2011).

BNYVV has a limited host range but a worldwide geographical distribution. In Lithuania it was first reported in 2004 (Jackeviciene *et al.*, 2005), but has not been investigated molecularly. Analysis of sequence variability of BNYVV isolates can reveal their geographical origin and allow determining the isolate type, which in turn can help predicting changes of virulence in the lo-

cal virus populations. Therefore, the present study aimed at analyzing the partial RNA-2 and RNA-3 sequences of Lithuanian BNYVV isolates and determining their genetic variability.

One hundred sixty five sugar beet root samples were collected from six Lithuanian sugar beet-growing districts (Kaunas, Kėdainiai, Marijampolė, Sakiai, Panevėžys and Vilkaviskis) from 2004 to 2009. Plant samples were assayed by DAS-ELISA (Clark and Adams, 1977) using a commercial kit (DSMZ, Germany).

Samples of sugar beet rootlets were used for RNA extraction using QuickPrep total RNA extraction kit (Amersham Biosciences, England) or TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RT-PCR was performed with a TProfessional thermocycler (Biometra, Germany) using specific primers for the CP and p25 genes (Schirmer *et al.*, 2005). First strand synthesis of viral cDNA was carried out using 2 µl of virus-specific reverse primer (20 µM) with 3 µl of total RNA and 6 µl of diethyl pyrocarbonate (DEPC)-treated water. The mixture was incubated at 70°C for 5 min and chilled on ice prior to the addition of 1.5 µl of DEPC-treated water, 2 µl of dNTPs (10 mM), 4 µl of MMLV RT 5X buffer, 0.5 µl ribonuclease inhibitor (40 U µl⁻¹) and 1 µl of MMLV reverse transcriptase (200 U µl⁻¹) (Fermentas, Lithuania). Reverse transcription was carried out at 42°C for 60 min and viral sequences were amplified using a special PCR mix [20 µM specific forward and reverse primers, 10X Taq reaction buffer, 25 mM MgCl₂, 10 mM dNTPs and 5 U µl⁻¹ Taq DNA polymerase (Fermentas, Lithuania)]. The PCR program used for BNYVV CP gene amplification was 94°C for 3 min, 35 cycles of 94°C for 1 min, 61°C for 1 min and 74°C for 1 min, followed by final extension at 72°C for 10 min. For p25 gene the conditions were 94°C for 3 min, 35 cycles at 94°C for 1 min, 64°C for 1.5 min and 72°C for 1 min, followed by final extension at 72°C for 10 min. All PCR reactions were carried out in 50 µl reaction volumes containing 46-45 µl PCR mix and 4-5 µl cDNA. PCR products were separated by electrophoresis on 1-2% agarose gels, stained with ethidium bromide and visualized in BioDocAnalyze gel documentation system (Biometra, Germany).

PCR products corresponding to the CP (567 bp) and p25 (719 bp) genes were purified using a DNA extraction kit (Fermentas, Lithuania) and sequenced by the DNA Sequencing Centre of the Institute of Biotechnology, (Vilnius, Lithuania), or at Macrogen (Korea). Sequencing was carried out in two directions and each sample was sequenced twice. Sequences were aligned using the DNASTAR program and analyzed with BLAST (NCBI), CLUSTAL W (Thompson *et al.*, 1994), and EMBOSS Transeq (<http://www.ebi.ac.uk/Tools/emboss/transeq/index.html>) programs. CLUSTAL W was used for multiple nucleotide and amino acid sequence alignments. Phylogenetic trees were constructed with

the MEGA 4.1 software (Tamura *et al.*, 2007), using the neighbour-joining method. The significance of branch order was estimated by 1,000 bootstrap replicates.

BNYVV isolates were identified in three different regions of the country: Sakiai, south-west (BNYVV-T), Kaunas, centre (BNYVV-St) and Panevėžys, north (BNYVV-Pn). To secure information on the diversity of these isolates, their types were identified by amplifying the CP gene. The amino acid (aa) residues 62, 103 and 172 of the 21 kDa CP were used to distinguish BNYVV A type isolates (T62, S103, L172) from those of B type (S62, N103, F172) (Kruse *et al.*, 1994; Koenig *et al.*, 1995; Miyanishi *et al.*, 1999). It is known that P type isolates are closely related to the A type isolates (Koenig and Lennefors, 2000; Meunier *et al.*, 2005; Schirmer *et al.*, 2005) but can be distinguished based on the aa residues R17 and I102 (Koenig *et al.*, 1995; Miyanishi *et al.*, 1999).

The CP sequence of three Lithuanian viral isolates (BNYVV-St, -T and -Pn) was determined (GenBank accession Nos. JF910093, JF910094 and JF910095). Two isolates (BNYVV-St, -T) were of B type and one (BNYVV-Pn) of A type. The CP of isolates BNYVV-St and -T was identical. The phylogenetic tree constructed using the CP aa sequences displayed three main groups, two of which comprising A type (groups I and II) and one (group III) B type isolates (Fig. 1, Table 1).

CP sequences within group I shared 100% identity, regardless of their geographical origin, except for the Italian isolate IV4 (Chiba *et al.*, 2011) which differed from other A type isolates due to G121D and R126G substitutions. This group included Lithuanian BNYVV-Pn isolates. Some isolates (all Chinese isolates) of this group contained RNA-5.

Group II consisted of P type isolates because most of them were of the A type with RNA-5 and contained the P type-specific CP aa residues (R17 and I102) (Koenig *et al.*, 1995; Miyanishi *et al.*, 1999). Phylogenetic analysis showed that no Lithuanian isolate clustered with P type isolates, whereas Iranian isolate IR-GR1 (Mehrvar *et al.*, 2009) was associated with P type group, although it did not contain RNA-5.

Group III included all European and Asian B type isolates. Within group IIIa none of the European isolates had the RNA-5. This group included the Lithuanian BNYVV-St and -T isolates, whereas group IIIb included just Asian isolates which contained RNA-5.

The p25 gene sequence of three Lithuanian isolates (BNYVV-St, -T and -Pn) was also determined (GenBank accession Nos. JF910101, JF910102 and JF910103). High protein sequence variability was detected particularly within a four aa tetrad (residues 67-70) in type A isolates (Schirmer *et al.*, 2005; Chiba *et al.*, 2008; Koenig *et al.*, 2008; Li *et al.*, 2008). The aforementioned aa tetrad is located downstream of a nuclear localization signal motif (Vetter *et al.*, 2004) and up-

stream of a zinc finger motif (Jupin *et al.*, 1992). Variations in these tetrads can have a strong influence on p25 oligomerization and on BNYVV pathogenicity in *Tetragonia expansa* (Klein *et al.*, 2007) and partially resistant sugar beet varieties (Liu and Lewellen, 2007; Acosta-Leal *et al.*, 2008; Chiba *et al.*, 2008).

The deduced p25 aa sequences showed that the BNYVV-St and -T isolates contained an AYHR tetrad (in position 67-70) while BNYVV-Pn contained an AHHG tetrad. Multiple p25 sequence alignments showed that sequences of the Lithuanian BNYVV B type isolates resemble those of French and German B type isolates, whereas the A type isolate (BNYVV-Pn) sequence resembles that of Belgian, Hungarian, Slovakian and Swiss BNYVV A type isolates (Fig. 2, Table 1). The phylogenetic tree constructed with the p25 sequence displayed three major groups, two of which comprising A type isolates (groups p25-I and p25-II) and one (group p25-III) the B type isolates (Fig. 2). According to the phylogenetic analysis, B type isolates

BNYVV-St and -T (this study) were assigned to the p25-III group together with the B type isolates containing an AYHR tetrad originating from Belgium, Germany, France and the Czech Republic. This was consistent with results of Schirmer *et al.* (2005) who found a correlation between CP, p25 and RNA-5 in the European BNYVV isolates.

The A type isolates identified on the basis of the CP sequence have multiple tetrad motifs in p25 and do not contain RNA-5, with a few exceptions. Isolates with a B type CP have a uniform tetrad motif AYHR and do not have RNA-5, whereas the P type CP-containing isolates have a SYHG tetrad and possess RNA-5. The A type BNYVV-Pn isolate (this study) was assigned to the p25 group I that contained mainly A type isolates from several countries (Spain, Netherlands, France, Italy, Belgium, Sweden, Turkey, United States and Hungary) and one B type Chinese isolate (CH2) with RNA-5.

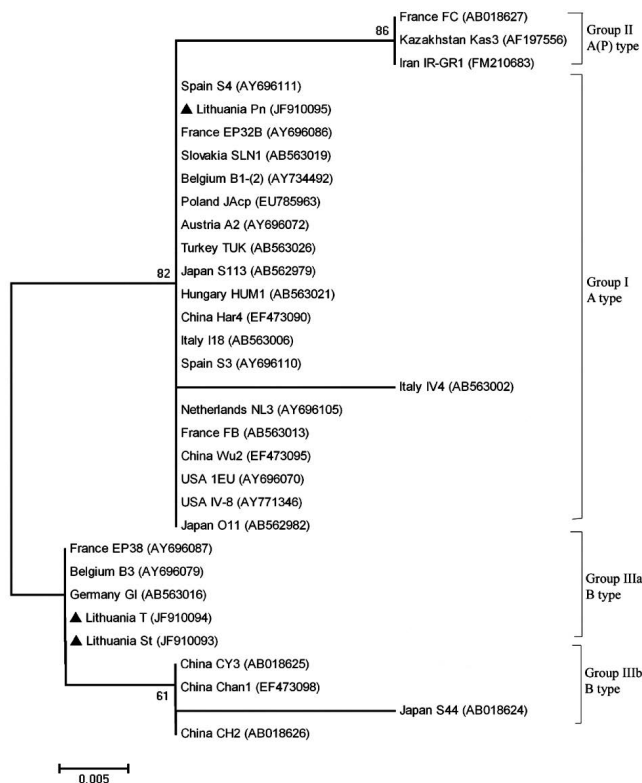


Fig. 1. Phylogenetic tree of *Beet necrotic yellow vein virus* RNA-2 encoded CP sequences, computed by MEGA 4.1 software, using the neighbour-joining method. The names indicate the country of origin and name of the isolate and are followed by accession number (in parentheses). Sequences determined in this study are marked with ▲. Scale bar indicates the phylogenetic similarity index (bar size refers to 0.005 amino acid changes per site). Numbers on branches represent the bootstrap values out of 1000 replicates. Only bootstrap values over 50 are shown.

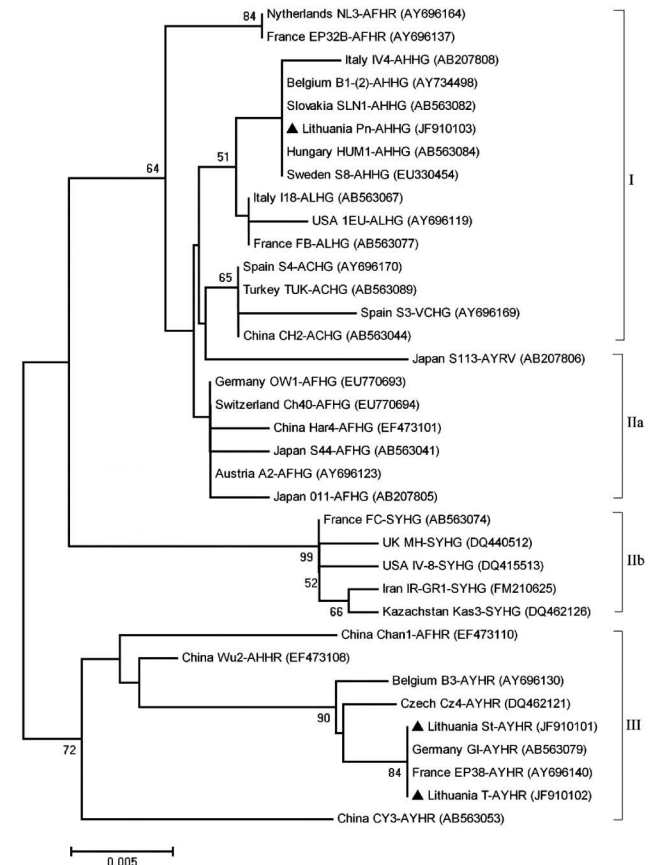


Fig. 2. Phylogenetic tree of *Beet necrotic yellow vein virus* RNA-3 encoded p25 sequences, computed by MEGA 4.1 software, using the neighbour-joining method. The names indicate the country of origin and name of the isolate. The tetrad of p25 protein is separated by a hyphen and is followed by accession number of the isolate (in parentheses). Sequences determined in this study are marked with ▲. Scale bar indicates the phylogenetic similarity index (bar size refers to 0.005 nucleotide changes per site). Numbers on branches represent the bootstrap values out of 1000 replicates. Only bootstrap values over 50 are shown.

Table 1. Geographical origin of *Beet necrotic yellow vein virus* (BNYVV) isolates and accession numbers of BNYVV RNA-2 encoded coat protein (CP), RNA-3-encoded 25 kDa protein (p25) genes.

Country	Virus isolate	Accession number		Amino acid tetrad (67-70 positions) of p25	CP type	Reference
		p25	CP			
Austria	A2	AY696123	AY696072	AFHG	A	Schirmer <i>et al.</i> , 2005
Belgium	B3	AY696130	AY696079	AYHR	B	Schirmer <i>et al.</i> , 2005
	B1-(2)	AY734498	AY734492	AHHG	A	Schirmer <i>et al.</i> , 2005
Czech Republic	Cz4	DQ462121	nd	AYHR	B	Ward <i>et al.</i> , 2007
Iran	IR-GR1	FM210625	FM210683	SYHG	A(P)	Mehrvar <i>et al.</i> , 2009
Spain	S3	AY696169	AY696110	VCHG	A	Schirmer <i>et al.</i> , 2005
	S4	AY696170	AY696111	ACHG	A	Schirmer <i>et al.</i> , 2005
Italia	IV4	AB207808	AB563002	AHHG	A	Chiba <i>et al.</i> , 2011
	I18	AB563067	AB563006	ALHG	A	Chiba <i>et al.</i> , 2011
	O11	AB207805	AB562982	AFHG	A	Chiba <i>et al.</i> , 2008, 2011
Japan	S44	AB563041	AB018624	AFHG	B	Miyanishi <i>et al.</i> , 1999
	S113	AB207806	AB562979	AYRV	A	Chiba <i>et al.</i> , 2008, 2011
USA	1EU	AY696119	AY696070	ALHG	A	Schirmer <i>et al.</i> , 2005
	IV-8	DQ41551	AY771346	SYHG	A	Liu and Lewellen, 2007
UK	MH	DQ440512	nd	SYHG	A(P)	Ward <i>et al.</i> , 2007
	Kas3	DQ462126	AF197556	SYHG	A(P)	Ward <i>et al.</i> , 2007
Kazakhstan	Kas3	DQ462126	AF197556	SYHG	A(P)	Koenig and Lennefors, 2008
	CY3	AB563053	AB018625	AYHR	B	Miyanishi <i>et al.</i> , 1999
China	CH2	AB563044	AB018626	ACHG	B	Chiba <i>et al.</i> , 2011
	Har4	EF473101	EF473090	AFHG	A	Miyanishi <i>et al.</i> , 1999
	Wu2	EF473108	EF473095	AHHR	A	Li <i>et al.</i> , 2008
	Chan1	EF473110	EF473098	AFHR	B	Li <i>et al.</i> , 2008
Netherlands	NL3	AY696164	AY696105	AFHR	A	Schirmer <i>et al.</i> , 2005
Hungary	HUM1	AB563084	AB563021	AHHG	A	Chiba <i>et al.</i> , 2011
	EP38	AY696140	AY696087	AYHR	B	Schirmer <i>et al.</i> , 2005
	EP32B	AY696137	AY696086	AFHR	A	Schirmer <i>et al.</i> , 2005
France	FC	AB563074	AB018627	SYHG	A(P)	Miyanishi <i>et al.</i> , 1999
	FB	AB563077	AB563013	ALHG	A	Chiba <i>et al.</i> , 2011
Slovakia	SLN1	AB563082	AB563019	AHHG	A	Chiba <i>et al.</i> , 2011
Sweden	S8	EU330454	nd	AHHG	A	Koenig <i>et al.</i> , 2008
Switzerland	Ch40	EU770694	nd	AFHG	A	Koenig <i>et al.</i> , 2008
Turkey	TUK	AB563089	AB563026	ACHG	A	Chiba <i>et al.</i> , 2011
Germany	G1	AB563079	AB563016	AYHR	B	Chiba <i>et al.</i> , 2011
	OW1	EU770693	nd	AFHG	A	Koenig <i>et al.</i> , 2008
Lithuania	St	JF910101	JF910093	AYHR	B	This study
	T	JF910102	JF910094	AYHR	B	This study
	Pn	JF910103	JF910095	AHHG	A	This study

A= A type CP; B= B type CP; (A)P= P type CP isolate; nd= not determined

No isolate investigated in this study was assigned to the p25-II group, which was divided into two sub-groups: IIa and IIb. P25-IIb contained only BNYVV A type isolates with p25 that carries a unique SYHG tetrad and contains RNA-5. Exceptions were the Iranian (IR-GR1) P type isolate and the American (IV-8) A type isolate, which did not contain RNA-5, but had a SYHG tetrad (Liu and Lewellen, 2007; Mehrvar *et al.*, 2009). Group p25-IIa contained A type BNYVV isolates mostly with an AFHG tetrad. Most east Asian isolates of this group contained RNA-5, except for the Japanese isolates S113 and S44. The German (OW1) isolate with RNA-5 belonged to this group, but its RNA-5 was more similar to the sequences of the east Asian than European

BNYVV isolates (Koenig *et al.*, 2008).

An interesting diversity within the p25 tetrad was observed with the Chinese BNYVV isolates assigned to the p25-III group (Fig. 2), one of which (Wu2) belongs to the A type with an AHHR tetrad whereas the other belongs to the B type (Chan1) with an AFHR tetrad. This indicates that the following isolates correspond to sequence variant intermediates from co-infected plant (Schirmer *et al.*, 2005; Li *et al.*, 2008). Chinese and Japanese isolates containing RNA-5 are associated with A and B type isolates and with a variable tetrad motif of p25. Thus, the east Asian isolates may share a common origin (Miyanishi *et al.*, 1999; Li *et al.*, 2008). Since the greatest diversity of BNYVV genomes was found in Chi-

nese and Japanese isolates, Chiba *et al.* (2011) recently suggested that BNYVV originally evolved in East Asia.

The survey for BNYVV has shown that isolates of the A and B types but no P type prevail in Lithuania, and that rhizomania is not widely spread in the country, its occurrence being limited to three regions. In Sakiai, where BNYVV was first recorded in 2004, sugar beets are no longer grown. Results of this study are important in terms of future efforts aiming at restraining the spread of BNYVV to new areas. As with other soil-borne viruses (Santala *et al.*, 2010), efforts to eradicate BNYVV from newly contaminated fields could be very difficult if not impossible without abandoning cultivation of susceptible crops for years. In areas where BNYVV occurs, introduction of rhizomania-resistant sugar beet cultivars obtained by means of traditional breeding or biotechnology (Zhang *et al.*, 2008) is the most important option whose sustainability depends on the genetic variability of the local BNYVV populations. This study provides valuable information for planning future strategies to control rhizomania in the Baltic region.

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