BIOLOGIA PLANTARUM 50 (4): 660-666, 2006

Variability in sequence of *Strawberry vein banding virus*

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Abstract

The variability of the *Strawberry vein banding virus* (SVBV) isolates was investigated. In total 267 strawberry plants from 6 European countries and North America were tested for the presence of SVBV. Only 4 plants were positive. Partial genomic sequences of the capsid protein gene of three North American SVBV isolates were determined. Only minor sequence variability (0.7 %) was observed during a comparison with existing nucleotide data of the European and the North American isolates (9 isolates). No variability at all could be found in the annealing regions of primers and probes used for molecular detection of SVBV for these isolates. However, a comparison to a sequence of a Chinese isolate published recently revealed a much higher DNA sequence difference (9.5 %) of this isolate.

Additional key words: caulimovirus, diversity, *Fragaria* sp., NASBA, PCR, quarantine.

Introduction

Strawberry vein banding virus is a member of the genus *Caulimovirus* infecting *Fragaria* species. Its presence has been reported from many countries worldwide: Australia, America, Asia, Africa (Converse 1992, Frazier and Morris 1987) and Europe (Czech Republic, Slovak Republic, Hungary and Serbia – Honetšlegrová *et al*. 1995, Petrzik *et al*. 1998b). SVBV has been listed as a quarantine pest by the European and Mediterranean Plant Protection Organization since 1978.

The characteristic symptoms in strawberry are chlorotic bands along the main leaf veins, streaking and spotting of older leaves and twisting of leaflets. Frazier and Morris (1987) reported an existence of two separate types of SVBV defined on basis of the symptoms caused in indicator strawberries – the *western type* typical by vein banding symptoms and the *eastern type* showing mainly rolling and curling of leaves. Little is known about the eastern type and all the isolates sequenced to date expressed the symptoms of vein banding (Mráz *et al*. 1998, Chun *et al*. 2003, Mahmoudpour 2003). Symptoms vary not only with virus isolate, but also with cultivar and the developmental status of the host, however, different strawberry cultivars are known to display a different reactivity to identical external factors (Ledesma *et al*. 2004). The SVBV symptoms range from almost latent infections to necrosis and severe stunting of whole plants. The most pronounced symptoms are often found in mixed infections with other strawberry viruses (Bolton 1974). The major viruses that share a common way of transmission with SVBV – the transmission by aphids – are the *Strawberry crinkle virus* (SCV), *Strawberry mottle virus* (SMoV) and *Strawberry mild yellow edge virus* (SMYEVP). A mixed infection of all these four viruses was recently described by Thompson *et al*. (2003) in wild strawberry plants from Chile.

Routine detection of SVBV for certification purposes still relies on time-consuming leaf-graft bioassays on indicator plants (Converse 1987). The development of modern molecular detection methods was enabled by the determination of the complete genomic sequence of SVBV by Petrzik *et al*. (1998a). It was found that the genome of SVBV is comprised of 7.876 base pairs long

Received 21 September 2004, *accepted* 24 May 2005.

Abbreviations: CaMV - *Cauliflower mosaic virus;* NASBA - nucleic acid sequence based amplification; PCR - polymerase chain reaction; SVBV - *Strawberry vein banding virus*.

Acknowledgements: This work was supported by a AV0Z50510513 grant and a 5th Framework grant from the EU No. QLRT-PL99-1553. Special thanks belong Dr. Robert Martin (USDA-ARS, Corvallis, USA) and Dr. Joseph Postman and Dr. Bruce Bartlett (the National Clonal Germplasm Repository, Corvallis, Oregon, USA) for the SVBV isolates provided. Field samples from the Czech and Slovak Republic and Hungary were kindly provided by Renata Karešová (RBIP, Hořice, Czech Republic) and Jana Fránová (IPMB, České Budějovice, Czech Republic). Field samples from Poland and Lithuania were provided by Miroslawa Cieslińska (IPF Skierniewice, Poland). The isolate from Germany was kindly provided by J.R Thompson (BBA, Institut für Pflanzenschutz im Ostbau, Dossenheim, Germany).

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double stranded circular DNA. Soon, a polymerase chain reaction (PCR) assay combined with hybridization to an SVBV-specific probe was developed by Mráz *et al*. (1997). Recently the PCR assay was optimized and a new nucleic acid sequence based amplification (NASBA) assay has been developed for SVBV (Vašková *et al*. 2004). Both the PCR and NASBA assay amplify the middle part of the capsid protein gene. Variability of this region was previously determined for one American and five European isolates (Mráz *et al*. 1998). Low variability in annealing regions of primers and probes is a crucial requirement for a reliable and robust performance of molecular techniques.

To obtain a more complete picture of the variability of SVBV, we supplement the complete and partial nucleotide sequences already available with additional sequences of SVBV isolates. In this work, the determination of sequence information of almost the entire capsid protein gene of three American SVBV isolates is reported. A comparison of symptoms and a previously observed level of variability of SVBV was made.

Materials and methods

Virus isolates: The North American SVBV isolates (9010, 9016, 9043, 9044 and 9093) used in this study were obtained from the National Clonal Germplasm Repository, Corvallis, Oregon, USA. They represent the American western-type isolates. The isolates were maintained on *Fragaria vesca* L. cv. Alpine and *Fragaria* × *ananassa* Duch. cv. UC4 and UC6. Isolate Lfp-Stuttgart-1996 form Germany was obtained from a virus collection of the BBA, Institut für Pflanzenschutz im Ostbau, Dossenheim, Germany. All plants were maintained in an insect proof air-conditioned greenhouse. The remaining 261 plants used for the large-scale testing were collected on fields and gardens preferentially during spring months. They originated from 6 European countries (Czech Republic – 216 plants, Slovak Republic – 29 plants, Hungary – 14 plants, Germany, Poland, Lithuania – each 1 plant). When only leaves were available, the material was used fresh (stored in cold for maximum 3 d) or frozen. When the whole plants were available, a fresh material was sampled just before the isolation.
Accession numbers of sequences published in this work: AY605662, AY605663, AY605664.

Isolation of DNA: Homogenization of strawberry leaves was done by means of the *Bioreba* (Reinach, Switzerland) extraction bags and the *Bioreba HOMEX 6* homogenizer in the sample extraction buffer (0.14 M NaCl, 2 mM KCl, 2 mM KH_2PO_4, 8 mM $Na_2HPO_4.2H_20$ (pH 7.4), 0.05 % (v/v) *Tween 20*, 2 % (m/v) polyvinylpyrrolidone 40, 0.2 % (m/v) ovalbumin, 0.5 % (m/v) bovine serum albumin, (m/v) 0.05 % sodium azide) added in a ratio 1:10 (m:v). This solution was used for extraction of DNA by the DNeasy Plant Mini Kit (*Qiagen GmbH*, Hilden, Germany). DNA was eluted in 0.2 cm^3 of low salt DNeasy dilution buffer and stored at -20 °C.

PCR: Primers annealed the capsid protein gene (nucleotides 1890-1910 and 3294-3313 of isolate with GenBank Acc. No. NC_001725) and the product of amplification was 1439 base pairs long. In total 0.1 μM primers (forward primer: 5' gct gaa ttc ATG GTA AGC AGA AGA GAA AGA C 3'; reverse primer: 5' aaa ggt acC TCC AGA TCT TCT GAG TC 3' non-viral nucleotides of attached restriction sites are shown in lower-case letters) were used in a standard PCR reaction (1x RedTaq PCR Reaction Buffer, 200 nM dNTP each, and 1 U RedTaq DNA polymerase). After initial denaturation at 94 °C for 10 min, the following cycle was repeated 35 times: 30 s at 94 °C, 30 s at 50 °C and 120 s at 72 °C. The final extension step lasted for 10 min at 72 °C. PCR products were analysed on a 1 % agarose gel.

Cloning: Product of the PCR reaction was ligated into the *pCR® 4-TOPO®* vector by means of the *TOPO TA* cloning Kit for sequencing. Then the *One Shot TOP10* chemically competent *Escherichia coli* cells were transformed by this vector according to instructions of the manufacturer (*Invitrogen GmbH*, Karlsruhe, Germany). Positive transformants were grown overnight in LB medium with ampicilin and the plasmids were isolated by the *GenElute™ Plasmid Miniprep* kit (*Sigma*, St. Louis, MI, USA).

Sequencing: The cloned PCR fragments were sequenced with primers T7 (5' TAA TAC GAC TCA CTA TAG GG 3'), T3 (5' ATT AAC CCT CAC TAA AGG GA 3'), RP (5' TTT CTC CAT GTA GGC TTT GA 3') and 34C9 (5' GCG CCA GCA ACC GAA GAC AAC T 3') by the *BigDye* terminator cycle sequencing ready reaction kit (*Perkin Elmer Applied Biosystems Division*, Foster City, CA) and analyzed on automated DNA *ABI PRISM 310* sequencer (*Perkin Elmer Applied Biosystems*, Lincoln, USA). All the sequences were read at least twice.

Post-sequencing analyses: Sequence data were read using the *CHROMAS* utility (*Technelysium Pty Ltd.*, Australia) and multiple sequence alignments were performed using the online service *CLUSTALW* (available from the European Bioinformatics Institute; Thompson *et al*. 1994). GenBank Acc. No. of sequences used in alignments are in Table 1. The published alignment was fitted by the program *BoxShade* (available online from the *Swiss EMBnet*).

Other methods used for SVBV detection: Most plants were tested by at least two methods simultaneously – it was the PCR (Vašková *et al*. 2004) complimented by either biological indexing or NASBA (Nucleic Acid Sequence Based Amplification). Biological indexing: leaves of the plants tested were grafted on indicator strawberries (*Fragaria ananassa* cv. UC4 and UC6). NASBA protocol was done as in Vašková *et al*. (2004).

Results and discussion

To investigate the genetic variability of SVBV and to ensure that possible difference between virus isolates will not hamper the newly developed detection methods, a collection of SVBV isolates was established. During the years 2000, 2001 and 2002, 261 plants with SVBV-like symptoms were tested in total. The plants originated from different European countries – preferentially from fields where SVBV was found before (Mráz *et al*. 1998, Petrzik *et al*. 1998b). Almost none of the plants showed to be SVBV-infected either by biological indexing, NASBA or by PCR. The only exception was an SVBV isolate originating in Germany. The plant tested positive for SVBV infection in early spring of 2001; however, later on in that year and the following years it was not possible to detect the virus again either in our laboratory, nor in the laboratory where the isolate originated from.

Fig. 1. SVBV isolates on indicator plants: *A* - isolate 9010, *B* - isolate 9093, *C* - isolate 9044.

Three of five SVBV isolates shipped from the National Clonal Germplasm Repository, Corvallis, Oregon, USA were positive in our hands. All the five isolates were tested by biological indexing and repeatedly also by NASBA and PCR.

All the three North American positive isolates (9010, 9044 and 9093) showed similar symptoms on indicator strawberry plants – yellow vein banding along the veins (Fig. 1). However, the time for symptoms to first appear was different for the particular isolates. All the plants were shipped to the Czech Republic in January 2002. Isolates 9010 and 9093 developed symptoms of SVBV as soon as in January 2002, while the plant infected with isolate 9044 did not develop any SVBV symptom till summer months of 2002. This plant also repeatedly tested negative in NASBA and PCR experiments during the spring months of 2002. In the summer months, it was both PCR and NASBA positive.

The sequence information of almost the entire capsid protein gene (1382 bp of 1426 bp) of the three North American SVBV isolates was determined. Variability among these isolates and the isolate with the Acc. No. NC_001725 (Stenger *et al*. 1988), for which the whole sequence was determined previously (Petrzik *et al*. 1998b), was 0.7 % (9 nt substitutions in a 1382 nt long region). This was similar to the variation observed by Mráz *et al*. (1998) who found 2 nt substitutions in a 430 nt long region (variability of 0.5 %). No variability was observed in the annealing regions of NASBA and PCR primers and probes comparing the American and European isolates. The recently reported Chinese isolate (Chun *et al*. 2003), however, showed a considerable variability throughout the whole sequenced segment of the capsid protein gene (Fig. 2).

Nucleotide and deduced amino acid sequences of SVBV exhibit a significant homology to corresponding sequences of the related *Carnation etched ring virus* (CERV) and *Cauliflower mosaic virus* (CaMV). For CERV, sequence of only one isolate was determined,

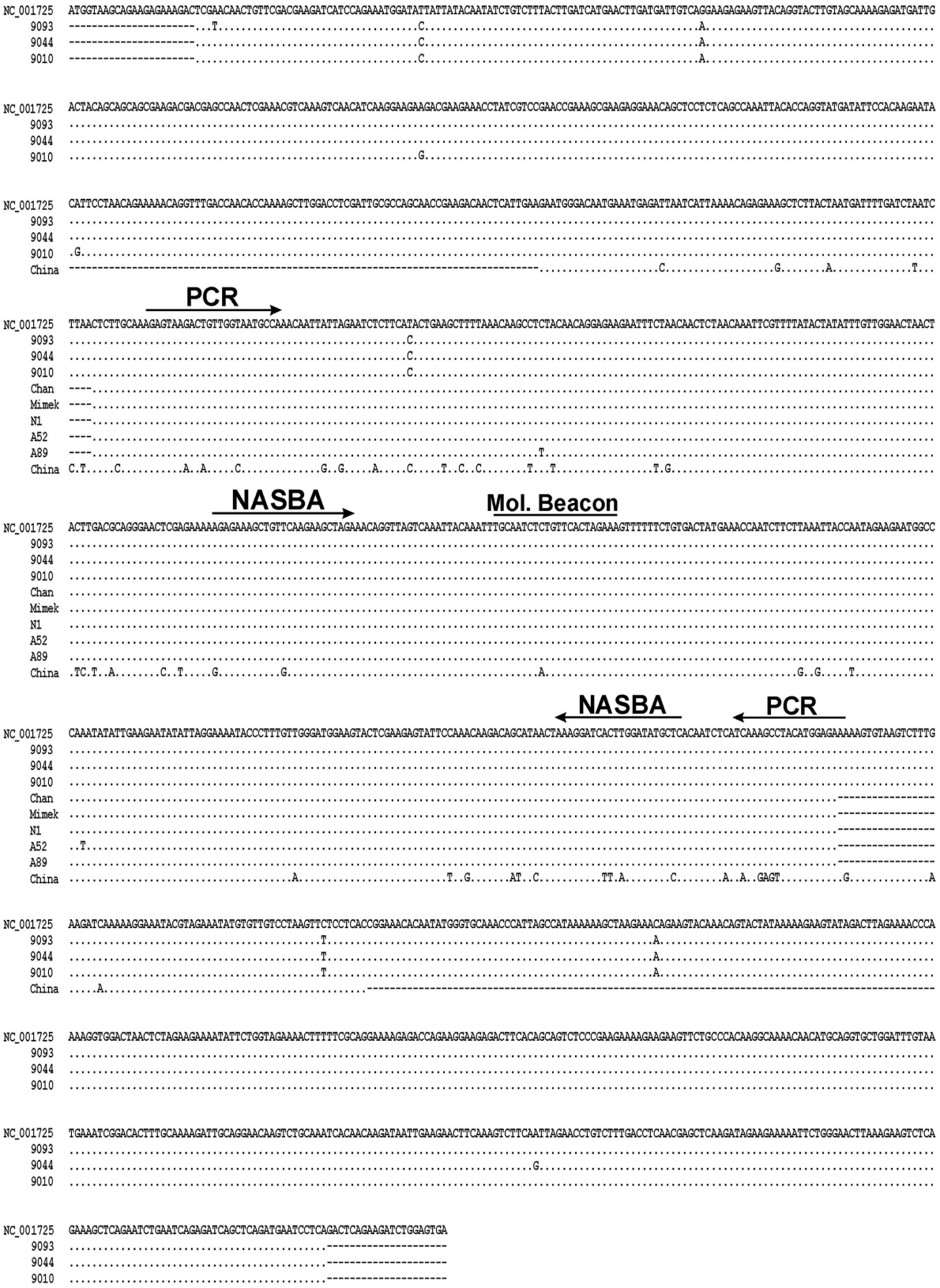

Fig. 2. Variability of the capsid protein gene of SVBV. (-) sequence not determined, (.) nucleotide identical to NC_001725, (→) anneling regions of NASBA and PCR primers and probes.

Table 1. Sequences used for alignments (* - *Blueberry red ringspot virus*, † - *Cestrum yellow leaf curling virus*, ‡ - *Figwort mosaic virus*, § - *Mirabilis mosaic virus*).

Acc. No.	Virus	Isolate	Country	Author
NC_001725	SVBV		USA	Petrzik *et al*. 1998a, Stenger *et al.* 1988
AY605662	SVBV	9093	Canada (British Colubia)	this work
AY605664	SVBV	9044	USA	this work
AY605663	SVBV	9010	USA	this work
–	SVBV	Chan (Chandler)	Germany	Mráz *et al*. 1998
–	SVBV	Mimek	Norway	Mráz *et al*. 1998
–	SVBV	N1	Czech Republic	Mráz *et al*. 1998
–	SVBV	A52	Czech Republic	Mráz *et al*. 1998
–	SVBV	A89	Czech Republic	Mráz *et al*. 1998
–	SVBV		China	Chun *et al*. 2003
NC_003498	CERV			Hull *et al*. 1986
NC_001497	CaMV			Franck *et al*. 1980
AF140604	CaMV	Xinjiang		Fang *et al*. 1985
V00140	CaMV	CM1841		Gardner *et al*. 1981
X79465	CaMV	B29		Pique *et al*. 1995
V00141	CaMV	Strasbourg		Franck *et al*. 1980
M90542	CaMV	BBC		Chenault and Melcher 1993a
M90541	CaMV	NY8153		Vaden and Melcher 1990
M10376	CaMV	Cabb-D/H		Balazs *et al*. 1982
M90543	CaMV	CMV-1		Chenault and Melcher 1993b
NC_003138	BRRV*		USA	Glasheen *et al.* 2002
NC_004324	CmYLCV†			Stavolone *et al*. 2003
NC_003554	FMV‡			Richins *et al*. 1987
AF454635	MMV§			Dey and Maiti 1999

so no calculation of its variability was possible. For CaMV however, 9 sequences of the capsid protein gene region were available (Table 1). Variability among these 9 isolates in the middle region of the capsid protein gene was 13.7 % (66 nt substitutions in a 480 nt long region). This number differed a lot from the variability of 9 European and American SVBV isolates in the corresponding region of the capsid protein gene, which was only 0.7 % (3 nt substitutions in a 430 nt long region).

The strawberry plant is a vegetatively propagated perennial and, therefore, the health control of the propagation material is important for its cultivation. The detection of SVBV is rather difficult, however. No commercially produced antibodies are available and thus no serological detection is possible. The only certified method for the moment is the time-consuming bioassay on indicator plants. Molecular methods based on the detection of the specific viral nucleic acids represent an important improvement of the situation. Their weak point is the variability of target sequences, however. To map the variability of the region chosen for the molecular detection of SVBV, partial sequences of all available SVBV isolates were determined. Only a minor sequence variability was revealed for SVBV, showing its homogeneity among isolates of different origins. This result is in a good concord with the previously published data (Mráz *et al*. 1998). A comparison to variability of the closely related CaMV showed notably different figures: 0.7 % for the variability of SVBV isolates in comparison with 13.7 % for CaMV isolates. The cause of this difference is not known yet, but characteristics like the host species spectra may provide some explanation. The natural host range for SVBV comprises only a few *Fragaria* species: *F. vesca*, *F. virginiana*, *F. chiloensis* and *F.* × *ananassa*. CaMV infects, on the other hand, a broad spectrum of species (*Brassicaceae*, *Resedaceae* and some species of the *Solanaceae* families). Also the spectrum of vectors of CaMV is much broader than that of SVBV. However, the geographic origin of SVBV isolates may also have some influence on the sequence variability of SVBV. The Chinese isolate sequenced recently (Chun *et al*. 2003) shows a 9.5 % sequence difference from the other sequenced SVBV isolates.

During the cultivation of SVBV infected plants some problems with the maintenance of SVBV in strawberry plants were encountered. It was the case of the German isolate kept in our greenhouse, as well as two isolates (9016 and 9043) kept in the National Clonal Germplasm Repository (NCGR). Dried strawberry leaves of isolates 9016 and 9043 that were obtained from the NCGR in 1995, were shown to be SVBV positive, as well as isolates 9010 and 9044. This result was obtained by Mráz *et al*. (1996) by hybridization of the nucleic acid extracts to an SVBV-specific probe. To our best knowledge, presence of SVBV in the isolates kept in the NCGR has not been tested in the last few years. In 2002 we could confirm the presence of SVBV only in three out of the five isolates.

The situation with isolate 9044 was rather

contradictory, too. The negative results of various tests performed during spring 2002 were in a sharp disharmony with the results of the tests performed during summer, autumn and winter months of the year 2002 and also with the reliability with which the isolates 9010 and 9093 were detected throughout the whole year. These data indicate that SVBV is rather difficult to maintain in experimental plants for longer periods and that much of its life cycle has not been revealed yet.

The incidence of SVBV seems to be rather low in Europe. During our extensive three-years-long survey, we could not confirm the presence of SVBV in five European countries, in spite of the large number of plants tested. Our effort to find new isolates remained without any success even in locations from which SVBV isolates were obtained in study of Mráz *et al*. (1997). The main reason is probably the use of certified healthy seed material in commercial plantations, which is more common now then it was ten years ago. Our data agree to data of Babini *et al*. (2004), who reported the absence of SVBV on strawberry plantations of Italy, Poland, Lithuania and Germany. Their data come from experiments with several hundered strawberry plants tested by biological indexing, PCR and/or NASBA.

In conclusion we obtained three new partial sequences of SVBV isolates and we confirmed a significant homogeneity of SVBV isolates of European and American origin.

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