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BRIEF REPORT

Molecular and serological characterization of an Iranian isolate of *Beet black scorch virus*

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Abstract An isolate of *Beet black scorch virus* (BBSV) was obtained from Iranian sugar beet roots. Its genome organization closely resembles that of the previously described Chinese and North American isolates, but the nucleotide sequences of the three isolates differ considerably. Most of the nucleotide exchanges, however, are silent, and the Iranian and the Chinese isolates were serologically indistinguishable. Beets infected by the Iranian BBSV did not show black scorch symptoms, but severe root beardedness. This might have been caused by BBSV or the simultaneously present beet necrotic yellow vein virus, or both together.

Beet black scorch virus (BBSV) was first described in China, where it was reported to cause severe black scorch symptoms on the leaves and necrosis in the roots of sugar beet, but no obvious root beardedness. It is transmitted in a non-persistent manner by *Olpidium brassicae*. Because of its morphological, biological, and molecular properties, it has been assigned to the genus *Necrovirus* in the family *Tombusviridae* [1, 2, 5, 9]. Recently, an isolate of BBSV with deviating molecular and possibly also pathogenic properties has been obtained in the USA from sugar beet plants showing severe rhizomania-like symptoms (root beardedness), but no black scorch symptoms [7, 8]. Many of these plants had tested negative for beet necrotic yellow vein virus (BNYVV) [7, 8], which is usually associated with rhizomania symptoms.

During a survey on the occurrence of rhizomania in Iran we have attempted to obtain a BNYVV isolate from root beards of sugar beet grown in the area of Torbet-Hydrieh in the northeast of the country by sap transmission to leaves of *Chenopodium quinoa*. The inoculated leaves developed severe necrosis already a few days after inoculation, and in a second passage many chlorotic local lesions, which soon turned necrotic, appeared already 3 days after inoculation. This suggested that a virus different from BNYVV had been isolated, because lesions produced by BNYVV usually need ca. 10 days to develop. Immunoelectron microscopy revealed in the *C. quinoa* leaves many isometric particles which were strongly decorated by an antiserum to the Chinese isolate of BBSV kindly provided by Dr. J. Yu.

The Iranian BBSV was purified by homogenizing 100 g of infected *C. quinoa* leaves in 300 ml 0.1 M sodium acetate, pH 6.0, containing 1% mercaptoethanol. The sap obtained after filtration through cheese cloth was stirred for 20 min at 4°C with an equal volume of a 1:1 (v/v) mixture of butanol and chloroform. The supernatant obtained after low-speed centrifugation was subjected to high-speed centrifugation through a 20% (w/v) sucrose cushion. The sedimented virus was further purified by cesium chloride density gradient centrifugation. An antiserum was produced in a rabbit by two intramuscular injections, 1 week apart, of virus emulsified in Freund's complete and incomplete adjuvant, respectively. Primers were designed on the basis of the published sequences of the Chinese and

Nucleotide sequence data reported are available in the GenBank database under the accession number EU545828.

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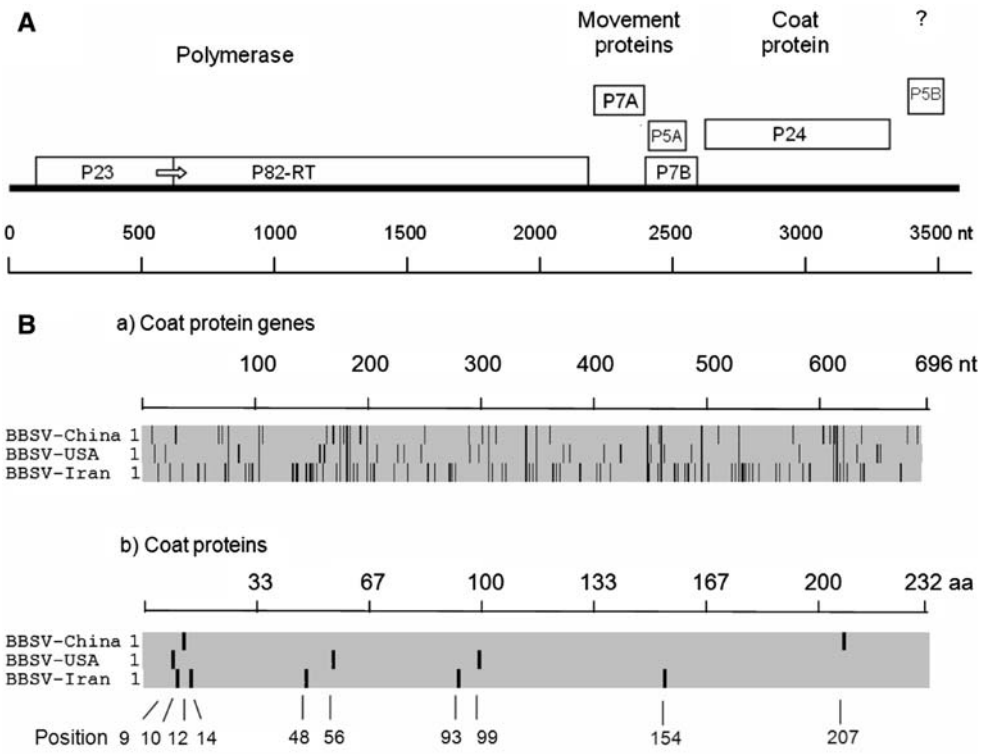

Fig. 1 Molecular properties of the Iranian isolate of BBSV. **A** Genome organization. **B** Alignments of the nucleotide sequences of the coat protein genes (**a**) and of the derived amino acid sequences of the coat proteins (**b**) of the three BBSV isolates sequenced so far. A *vertical black bar* signifies that the nucleotide or amino acid in the indicated position differs from those in the corresponding position in the coat protein gene or the coat protein, respectively, of the other two virus strains

American BBSV isolates in conserved regions to produce PCR products of overlapping sequences from which almost the entire nucleotide sequence of the genomic RNA of the Iranian BBSV isolate, except for the 5′- and 3′-terminal primer regions, was assembled. The sequencing was done by a commercial company (MWG-Biotech, D85560 Ebersberg, Germany). The sequences were assembled and analyzed by means of the Invitrogen Vector NTI Advance 10 program. For the routine detection of BBSV by means of RT-PCR, we used sense and antisense primers derived from nts 3,341 to 3,360 and 3,604 to 3,625, respectively, of the Chinese BBSV sequences (accession nos. AF452884 and AY626780). These sequences are also conserved in the RNA of the US isolate of BBSV (accession no. EF153268).

The genome organization of the Iranian BBSV isolate (Fig. 1A) closely resembles that of the Chinese and American BBSV isolates. The 5′-proximal ORF 1, which encodes a 23-kDa protein, is extended into a second ORF (ORF 2) by readthrough of an amber stop codon to encode a 82-kDa readthrough protein. As previously shown for the Chinese and the US BBSV isolates [2, 8], this protein carries a GDD motif typically occurring in RNA-dependent RNA polymerases. In addition to this triad, sequence stretches which were very similar to the eight RNA-dependent RNA polymerase motifs described for tomato bushy stunt virus (TBSV) [4] were readily detected in P82 of the Iranian BBSV isolate (Table 1) and also in the Chinese and the US isolates (not shown). TBSV is a tombusvirus, which like the

Table 1 Comparison of the eight RNA-dependent RNA polymerase sequence motifs described for tomato bushy stunt virus (TBSV) [4] with corresponding areas in the 82-kDa protein of the Iranian isolate of BBSV

Sequence motif no.	TBSV (NC_001554)	BBSV-Iran (EU545828)
I	FVKAEK (448–453)	FVKAEK (348–353)
II	VIQPRNPRYNVELGRYLRHMESKLMKAVDG (465–494)	VIQPRSPRYNVEVGRFLRHAEEHLFDAINR (366–395)
III	IKGYTADEVGAIF (502–514)	FKGLNADQAGMEM (403–415)
IV	LDASRFDQHCS (529–539)	MDASRFDQHVS (430–440)
V	EGCRMSGDINTSLGNYLLMCAMVHGYM (586–612)	EGCRMSGDINTSSGNCYIMCATVHNYC (489–515)
VI	LANC**GDD** CVL (622–631)	LANN**GDD** CML (525–534)
VII	EEVEFCQAH (666–674)	EHLEFCQTR (569–577)
VIII	WKMVRNVRTA (682–691)	YRMVRNLHQG (585–594)

The positions of the motifs in the respective amino acid sequences are given in parentheses; identical amino acids in the sequences of the two viruses are underlined. The GDD triad in motif VI is highlighted by bold letters

necroviruses, belongs to the family *Tombusviridae* [5]. As in the Chinese and the US BBSV genomic RNAs, three small ORFs (ORF 3, 4, and 5) coding for proteins p7A, p7B, and p5A, respectively, are found in the central part of the Iranian BBSV RNA. ORF 5 is nested within ORF 4 but is in a different frame. With the Chinese isolate, all three small ORFs have been found to be necessary for cell-to-cell movement [9]. The sixth ORF, with the coding capacity for a 24-kDa protein, is assumed to be the coat protein gene. For the coat proteins of the Chinese and the US isolates a molecular mass of 24 kDa has been determined in SDS polyacrylamide gel electrophoresis [2, 8]. In addition, the location of ORF 6 corresponds to that of the coat protein genes of other necroviruses [5, 6]. In the RNA of the Iranian BBSV isolate only, a small ORF for a 5.3-kDa protein (p5B) is found downstream of the coat protein gene. Its TAG stop codon is also present in the sequences of the Chinese and US isolates, which, however, lack the ATG start codon. This small ORF shows no appreciable sequence identity with the putative p7 gene downstream of the coat protein gene in tobacco necrosis virus A, another necrovirus [6]; it may not be expressed. Due to an internal stop codon, the genomic RNA of the Iranian BBSV lacks the additional small ORFs originating at the 3′ end of ORF 2 in the Chinese and the US BBSV isolates [2, 8, 9]. With the Chinese isolate, this small ORF has previously been concluded to represent a cryptic, non-essential gene [9]. The ORF for a 10-kDa protein which is nested in the coat protein gene of the US isolate [8] is in the RNAs of the Iranian as well as the Chinese isolates, disrupted by an internal stop codon. Common features which differentiate the genomes of all three BBSV isolates from those of other necroviruses are the existence of the small p5A ORF (ORF 5) nested in a different frame within the p7B ORF (ORF 4) and the smaller size of the coat protein gene. All other necroviruses analyzed so far have coat proteins with molecular masses around 29–30 kDa [5]. We failed to detect a satellite RNA in Iranian BBSV using primers derived from the sequence of the satellite RNA detected in one of the Chinese isolates of BBSV [3].

Sequence comparisons revealed that there are many nucleotide differences between the genomic RNAs of the three BBSV isolates. The Iranian isolate is more distantly related to the Chinese and the US isolates than these two isolates are to each other (Table 2). Nucleotide variation is found along the entire sequence of the genomic RNAs and is especially pronounced in the coat protein gene, as has previously been noticed already for the US isolate [8] (Table 2, Fig. 1B-a). Many of the nucleotide changes are, however, silent; thus the variation is much lower on the amino acid level (Table 2, Fig. 1B-b). This suggests that the three BBSV isolates analyzed may have separated during evolution a long time ago. There must have been, however, a strong selection pressure to retain the original protein structure.

The few differences which are found in the coat protein amino acid chains (Fig. 1B-b) apparently have no influence on the antigenic properties of the virus particles. In agar gel double diffusion tests, the Iranian isolate reacted strongly not only with its own antiserum but also with antisera to the other two isolates. When the Chinese and the Iranian isolates were placed in adjacent wells in agar plates, the precipitin lines formed with each of the three antisera fused without spur formation (results not shown), indicating that the Chinese and the Iranian isolates are serologically indistinguishable. Due to quarantine regulations, the US isolate could not be included in these tests. Since its antiserum reacted strongly with the two other isolates and its coat protein differs only in three amino acid positions from those of the other isolates (Fig. 1B-b), it is very likely to be serologically closely related or even identical to these isolates.

In an attempt to obtain information on possible further occurrences of BBSV in various parts of the world, antiserum to the Iranian BBSV was used for an ELISA with sugar beet root samples which were received previously from areas where severe rhizomania symptoms had been observed. Most of the samples were air dried and had been stored for up to several years at 4°C. Thirty-six samples originated from Iran, 11 from East Asia (China, Japan) and

Table 2 Comparisons of nucleotide and amino acid sequences of the BBSV isolates from Iran, China, and the USA

	Percentages of sequence substitutions in comparisons of BBSV originating from		
	Iran and China	Iran and USA	China and USA
Total RNA (nt)	11.1	11.1	6.5
82-kDa polymerase coding sequence (nt)	10.4	10.5	6.9
Coat protein gene (nt)	15.4	15.2	8.8
82-kDa polymerase amino acid sequence	2.5	3.3	2.5
Coat protein amino acid sequence	3.0	3.4	2.2

The GenBank accession numbers are EU545828, AF452884, and EF153268 for the Iranian, Chinese (Ningxia), and US (Colorado) isolates, respectively. The Chinese Xinjiang isolate (AY626780) was not included because it shares 99.45% sequence identity with the Chinese Ningxia isolate

124 from various European countries (Austria, Belgium, the Czech Republic, Denmark, England, France, Germany, Greece, Hungary, Italy, The Netherlands, Slovakia, Spain, and Switzerland). A positive ELISA result, which was readily confirmed by immunoelectron microscopy but not by RT-PCR, was obtained only with one out of four aliquots of a root sample from one European country (code no. EU 323). Also, only three out of four aliquots of the Iranian root sample from which our isolate was obtained tested positive in ELISA. These inconsistencies in the detectability of the virus may be due to an uneven distribution of the virus in various parts of a root sample and/or to the fact that in necrotic and possibly deteriorating tissue the virus may no longer be detectable. Root necrosis has been described as a symptom in beets infected by the Chinese BBSV isolate [2]. For these reasons, we cannot exclude the possibility that further samples which had tested negative in our ELISA might actually have contained the virus.

Leaves of the beet plants from which the Iranian BSBV was obtained, like those from which the US isolate [8] was obtained, did not show black scorch symptoms. Our plants, did, however, exhibit pronounced root beardedness like the beets infected by the US isolate did [8]. However, whereas most of the US plants did not contain detectable amounts of BNYVV, this virus was readily detected in the Iranian beets. The Iranian BBSV may or may not have contributed to the severity of the rhizomania symptoms. Further research will be necessary to study possibly existing differences in the pathogenicity of various BBSV isolates and to improve the reliability of virus detection, for example, by examining different parts of freshly harvested beets plants.

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