Occurrence of *Beet black scorch virus* Infecting Sugar Beet in Europe

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ABSTRACT

González-Vázquez, M., Ayala, J., García-Arenal, F., and Fraile, A. 2009. Occurrence of *Beet black scorch virus* infecting sugar beet in Europe. *Plant Dis.* 93:21-24.

In a survey of soilborne viruses infecting sugar beet in central Spain, *Beet black scorch virus* (BBSV) was detected in field grown sugar beets with symptoms of rhizomania disease. BBSV was found in all analyzed sugar beet producing regions from central Spain, as well as in bait plants grown in soils with a history of rhizomania from several Western European countries, thereby constituting the first report of BBSV in Europe. BBSV was transferred to *Chenopodium quinoa*, where it caused chlorotic local lesions from which virus particles were purified. The nucleotide sequence of the 3'-untranslated region of the genomic RNA was determined for 13 European isolates, and sequences were highly similar to those reported for Chinese and U.S. isolates. Sequence comparisons revealed three clusters of sequences, one including most European isolates, one including one European and two Chinese isolates, and the third including the U.S. isolate. BBSV was detected in a number of samples with rhizomania symptoms in which *Beet necrotic yellow vein virus* went undetected. However, its role in rhizomania disease in Europe, if any, remains to be established.

Additional keywords: Necroviruses

Diseases caused by soilborne viruses, particularly by those persistently transmitted by plasmodiophorid and chytrid vectors, are a serious threat to field crops worldwide. This is the case of rhizomania of sugar beet, caused by the benyvirus *Beet necrotic yellow vein virus* (BNYVV), which is persistently transmitted by the plasmodiophorid *Polymyxa betae* (18). Rhizomania is a limiting factor for sugar beet production, and in soils infested by BNYVV and its vector, only resistant cultivars can be grown with an acceptable yield. Sources of resistance to BNYVV have been found in *Beta vulgaris* subsp. *maritima*, and cultivars of sugar beet partially resistant to BNYVV have been developed by introgression of the genes *Rz1* and *Rz2* (7). Although these resistant cultivars give adequate protection in most regions, there is serious concern about the possibility of the appearance and dispersion of BNYVV strains able to overcome the existing resistance factors. Until now, BNYVV isolates able to cause rhizomania symptoms on resistant (*Rz1*) cultivars have

been reported in Europe and Asia, associated with the presence of satellite-like RNA5 (6,10,11). In the United States, the ability to overcome the same resistance appears to be associated with mutations in the RNA3-encoded p25 protein (1,14,15).

Other *P. betae*-transmitted viruses infecting sugar beet are *Beet soil-borne virus* (BSBV) and *Beet virus Q* (BVQ), both belonging to the genus *Pomovirus*, which have been reported in Europe, including Spain (12,13,17,20), and *Beet soil-borne mosaic virus* (genus *Benyvirus*), which has only been reported in North America (18). These viruses have no demonstrated role in rhizomania disease (16,18). In addition, a sugar beet-infecting virus, *Beet black scorch virus* (BBSV, genus *Necrovirus*, family *Tombusviridae*), was reported in China, and two isolates have been characterized molecularly (3,4). BBSV is transmitted by *Olpidium brassicae* zoospores (8) and is reported to cause black scorch of leaves and necrosis of roots (2,5). In China, a satellite RNA (BBSV-satRNA) has been found associated with BBSV isolates from Xinjian Province (5), but it has not been found in association with BBSV from other areas. Recently, BBSV has been reported in the United States, isolated from sugar beet plants with rhizomania-like symptoms, and a U.S. isolate has been molecularly characterized (22,23).

In Spain, rhizomania is an important disease of sugar beet crops in the northern

and southern central plateau, where beet is planted in spring and harvested in winter. Resistant cultivars account for greater than 90% of the crop in these areas, and usually perform well both in the presence and in the absence of rhizomania disease. During the last 3 years in some areas of the southern central plateau, however, rhizomania-like symptoms were apparent in BNYVV-resistant cultivars. This prompted us to re-analyze the distribution of BNYVV and of other soilborne viruses infecting sugar beet in Spain. Information on the distribution of BBSV and BVQ in Spain was available (17), but this was not the case for BBSV. In the course of this survey, BBSV was detected in sugar beets from Spain, as well as in soils from other European countries with a history of sugar beet production. Thus, we report here the first detection of BBSV in Europe. Comparison of the nucleotide sequence of the 3'-untranslated region (3'-UTR) suggests at least three clusters of BBSV isolates worldwide.

MATERIALS AND METHODS

Samples. Field-grown sugar beet plants with rhizomania symptoms from different locations in central Spain were collected, and sugar beet soils were obtained from different European countries, all with a history of rhizomania. The origins of Spanish sugar beet plants and European soils are described in Table 1.

Two 200-ml (6 cm diameter) pots were filled with a sample of each of the European soils, and five sugar beet seedlings cv. Orbis (Strube-Dieckmann, Germany) were planted in each pot. The cultivar Orbis is registered in Spain as a genetic monogerm and is susceptible to rhizomania. This variety has been used by AIM-CRA (Asociación de Investigación y Mejora del Cultivo de la Remolacha Azucarera, private sugar beet research institute) in field assays for the past 8 years as a susceptible control in tests for resistance to rhizomania because of its susceptibility and adaptation to Spanish conditions. The pots were placed in separate trays for each sample set, and the soil was kept moist during the 2 months prior to sampling.

Detection of BNYVV and BBSV. The lateral roots of sugar beet or roots of bait plants were washed with running tap water, the rootlets were collected, and total

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Accepted for publication 9 September 2008.

nucleic acids (TNA) were extracted from 200 mg of rootlets using Tri Reagent (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's protocol. The presence of BNYVV and of BBSV was analyzed by reverse transcription–polymerase chain reaction (RT-PCR). For RT-PCR detection of BNYVV, the primers described in Meunier et al. (16), which amplify a 545-bp fragment from RNA2, were used. For BBSV detection, the primers used were identical to nucleotides

3341–3370 of BBSV genomic RNA (BBSV3UTRfwd) and complementary to the 3' terminal 27 nucleotides (positions 3618–3644) (BBSV3rev) (nucleotide position numbers refer to sequence AF452884), as suggested by J. Weiland (*personal communication*). The primers amplify a 303-bp fragment including the 299 nt 3'-UTR of BBSV-RNA. For detection of BBSV, sat RNA primers BB-S3 and BB-S7 as described in Guo et al. (5) were employed. Positive controls were sugar

beet roots experimentally infected with BNYVV pathotype A and a plasmid containing the 3'-UTR of the U.S. isolate of BBSV, the kind gifts of Claude Bragard (Université Catholique de Louvain, Louvain-la-Neuve, Belgium) and J. Weiland (USDA-ARS, Fargo, ND, USA), respectively.

BBSV sequence. The nucleotide sequence of the 3'-UTR of BBSV RNA was determined from the RT-PCR amplicons obtained with primers BBSV3UTRfwd and BBSV3rev (Secugen S.L., Madrid, Spain). The 3'-UTR nucleotide sequence was determined for all the isolates from soil samples of other European countries that were positive for BBSV, as well as for three randomly selected isolates from each of the two sugar beet–growing areas of central Spain. The resulting sequences, together with those available from published isolates (accession numbers AY626780, AF452884, and EF153268), were aligned using CLUSTAL X (21). Genetic distances between sequences were obtained using Kimura's two parameter method (9), and the distance matrix was used to determine relationships among the isolates using the neighbor-joining method (19).

BBSV characterization. BBSV from field-infected sugar beet roots was purified according to Guo et al. (5) with modifications: 50 g of washed roots were ground at 4°C in 2 volumes of 0.1 M phosphate buffer, pH 7.0, 1 volume of chloroform, and 1% 2-mercaptoethanol. The slurry was centrifuged at 7,900 × g for 10 min, and the supernatant was centrifuged at 145,000 × g for 90 min. The pellet was resuspended overnight in 0.02 M phosphate buffer, pH 7.0. The resulting preparation was used to inoculate *Chenopodium quinoa* leaves. Four days after inoculation, BBSV particles

Table 1. Origin of the samples tested and presence of *Beet necrotic yellow vein virus* (BNYVV) and *Beet black scorch virus* (BBSV) in the roots of sugar beets sown in different soils

Origin	Sample	Presence of ^a	
		BNYVV	BBSV
North central Spain			
Pampliega (Burgos)	Bu-Pm	0	2
Laguna de Negrillos (León)	Le-LN	1	1
Pobladora (León)	Le-Pb	0	1
Nava del Rey (Valladolid)	Va-NR	0	2
Laguna (Valladolid)	Va-Lg	0	4
Bercero (Valladolid)	Va-Be	0	5
South central Spain			
Daimiel (Ciudad Real)	CR-Dm1	2	1
Daimiel (Ciudad Real)	CR-Dm2	4	1
Aguas Nuevas (Albacete)	Ab-AN	1	1
Minaya (Albacete)	Ab-My	1	1
La Roda (Albacete)	Ab-Rd	3	0
Europe			
United Kingdom B	GB-B	1/1	1/1
United Kingdom K	GB-K	2/2	2/2
Italy C	IT-C	0/3	1/2
Italy CP	IT-CP	0/3	1/3
France	FR2	1/1	1/1
Germany	D1	2/3	3/3
The Netherlands	NL1	3/3	2/3

^a For samples from Spain, data are number of positive plants in reverse transcription–polymerase chain reaction (RT-PCR) analyses per five tested plants per site. For European soils, data indicate the reaction, positive or negative, in RT-PCR, in extracts of five pooled plants per pot per number of analyzed plots. The number of pots varied with soil availability. RT-PCR for BBSV and BNYVV were done using the primers described in Materials and Methods.

Fig. 1. Detection and characterization of *Beet black scorch virus* (BBSV) from Europe. **A**, Reverse transcription–polymerase chain reaction (RT-PCR) with primers for BBSV detection in nucleic acid extracts from beet samples from Spain (1: Va-Be, 2: CR-Dm1, 3: Le-LN, and 6: Ab-Rd) and other European countries (4: IT-CP, 5: GB-B). 7: Positive control. **B**, Local lesions in *Chenopodium quinoa* leaves at 4 days postinoculation with virion preparations from sugar beet roots of sample Va-Be. **C**, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of proteins in viral particles purified from *C. quinoa* leaves. Unstained SDS-PAGE standards (left) were: 97.4 kDa: Phosphorylase b; 66.2 kDa: Bovine serum albumin; 45 kDa: Ovalbumin; 31 kDa: Carbonic anhydrase; 21.5 kDa: Trypsin inhibitor; 14.4 kDa: Lysozyme.

were purified from *C. quinoa* leaves, virion RNA was extracted in 0.02 M Tris, pH 8.5, 0.02 M NaCl, 5 mM EDTA, 1% sodium dodecyl sulfate (SDS), maintained at 60°C for 5 min, and extracted twice with phenol:chloroform (1:1). The RNA was used as a template for RT-PCR with the primers used for BBSV detection (BBSV3UTRfwd and BBSV3rev). To determine the molecular weight (MW) of the capsid protein, purified BBSV particles from *C. quinoa* were disrupted in 50 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue at 95°C for 3 min, and proteins were analyzed by denaturing 12% SDS–polyacrylamide gel electrophoresis (PAGE) and staining with Coomassie Brilliant Blue R-250. To estimate the MW of the protein, unstained SDS-PAGE standards were used (Bio-Rad, Hercules, CA, USA).

RESULTS AND DISCUSSION

BNYVV was detected in roots of field-grown plants from all analyzed sites in

southern central Spain, and from one out of six analyzed sites in northern central Spain (Table 1). BNYVV was also detected in roots from bait plants grown in soils from various European countries, except those from Casale and Copparo (Italy) (Table 1). BBSV was detected in most analyzed samples, with the exception of those from La Roda (Albacete, Spain) (Table 1), showing the widespread distribution of this virus in sugar beet growing areas from central Spain and, possibly, in the rest of the European countries from which soils were analyzed. In all cases, RT-PCR resulted in the amplification of a band of 303 bp, as expected from the nucleotide sequence of BBSV RNA from China (Fig. 1A). BBSV-satRNA was not detected in any of the samples that were positive for BBSV (not shown).

The nucleotide sequence of the 3'-UTR of BBSV RNA was determined from the RT-PCR amplicons for three randomly chosen sites in northern central Spain (Le-Pb, Acc. No. AM941660; Le-LN,

Acc. No. AM941659; and Va-Be, Acc. No. AM941661), three randomly chosen sites in southern central Spain (CR-Dm1, Acc. No. AM941662; CR-Dm2, Acc. No. AM941663; and Ab-My, Acc. No. AM941658), and for all sites in other European countries where the virus was detected (samples D1, Acc. No. AM941669; NL1, Acc. No. AM941670; GB-B, Acc. No. AM941664; GB-K, Acc. No. AM941665; Fr-2, Acc. No. AM941668; IT-C, Acc. No. AM941666; and IT-CP, Acc. No. AM941667). The comparison between the nucleotide sequences (not shown) revealed limited nucleotide diversity, with nucleotide identity $\geq 97\%$. It should be pointed out that this degree of nucleotide identity is within the range of that between the reported sequences for three BBSV isolates, two from Ningxia and Xingjiang provinces in China (accession numbers AY626780 and AF452884) and one from the United States (accession number EF153268), which is 97.32%, suggesting that the 3'-UTR is

Fig. 2. Neighbor-joining tree for sequences of the 3'-untranslated region (3'-UTR) of *Beet black scorch virus* (BBSV) isolates from Europe. The significance of the nodes in a bootstrap analysis with 1,000 replicates is shown. 3'-UTR sequences from U.S. (EF153268) and Chinese (AF452884, AY626780) isolates are identified by their origin and EMBL data bank accession numbers.

highly conserved in BBSV RNA. The dendrogram resulting from the analysis of the 3'-UTR sequences by the neighbor-joining method (19) showed three significant clusters (Fig. 2): one included the two sequences from China and the sequence from one site in southern central Spain (CR-Dm2), a second cluster included the sequence from the United States, and a third included most sequences from Europe. Thus, on the basis of the 3'-UTR, three putative strains can be defined for BBSV, which we name here the European, U.S., and Chinese. Comparison of 299 nt within the 3'-UTR of the three strains identified 19 polymorphic positions. Four of these are specifically associated with the U.S. strain, four are associated with the Chinese strain, and one is associated with the European strain. This means that between the putative U.S. and Chinese strains, there are 8 specific differences, and 5 specific differences between the European and U.S. or the European and Chinese putative strains. Nucleotide identities within each strain were greater than 99% within this region of the genome. Both European and Chinese strains are present in Europe, with an apparent higher prevalence of the first strain based on data presented herein.

To partially characterize a BBSV isolate from Europe, BBSV from sample Va-Be was chosen. Sugar beet roots positive for BBSV from this site were negative for BNYVV. BBSV purified from field-infected sugar beet roots was used to inoculate *C. quinoa* leaves. After 4 days in a greenhouse at 20 to 25°C and a 16-h light period, inoculated leaves showed abundant chlorotic local lesions (Fig. 1B); at this time, no systemic symptoms were observed. Nucleic acid extracts from infected *C. quinoa* leaves were negative for BNYVV, as well as for BSBV and BVQ (not shown), and positive for BBSV in RT-PCR assays. Viral RNA was extracted from BBSV particles purified from *C. quinoa* leaves, and was used as a template for RT-PCR with the primers described previously for BBSV detection. The nucleotide sequence from the amplified fragment was 100% identical to that obtained from the nucleic acid extracts from the field-infected sugar beet (not shown). Disrupted purified BBSV particles showed a single band with an apparent molecular weight around 21 kDa when analyzed by SDS-PAGE under reducing conditions (Fig. 1C), which is in line with the molecular size deduced for the 24.4-kDa coat protein (CP) from the nucleotide sequences of Chinese and U.S. isolates (23). Interestingly, oligonucleotide primers based on previously reported BBSV sequences have failed to amplify the CP gene of any "isolate" from Europe (not shown), suggesting a higher diversity at this genomic region, in agreement with the observation that this

region has the lowest level of nucleotide sequence conservation in open reading frame (ORF) comparisons between BBSV isolates (23). The nucleotide identities in the CP ORF for the three BBSV sequences in the GenBank database are 99.6% between the two Chinese isolates and 91% between the Chinese and U.S. isolates, the latter of which are the lowest genetic conservation for any ORF of BBSV.

In summary, our results show that BBSV, unreported until now in Europe, is widespread in soils of Western Europe with a history of sugar beet cultivation. Two putative strains of BBSV appear to be present in Europe, based on comparison of sequences within the 3'-UTR sequence, albeit with broadly different prevalence. The U.S. isolate of BBSV was obtained from sugar beet plants with rhizomania-like symptoms from which BNYVV and BSBMV were not detected (23). Similarly, in this work, BBSV was detected in roots of European sugar beet plants with rhizomania-like symptoms from which BNYVV was also unable to be detected. The presence of BBSV in association with these symptoms does not indicate any clear role for BBSV in inducing rhizomania-like symptoms, and further research will be necessary to clarify the nature of this association.

ACKNOWLEDGMENTS

This work was in part supported by contract P070210-071 between AIMCRA and Departamento de Biotecnología, UPM. We thank Mike Asher (Broom's Barn Research Centre, UK), Hans Schneider (Institute of Sugar Beet Research-IRS, The Netherlands), Mark Varelmann (Institut für Zuckerrübenforschung – IFZ, Germany), Marc Richard-Molard (Institut Technique Français de la Betterave Industrielle-ITB, France), and Nicola Minerva (Società Italiana per la ricerca e la sperimentazione in Bieticoltura - Beta S.C.ar. L., Italy) for sending soil samples, and Claude Bragard and J. Weiland for providing positive controls for the detection of BNYVV and BBSV. Begoña Prieto provided excellent technical assistance.

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