NOTE
Virology / Virologie

Isolation, partial sequencing, and phylogenetic analyses of *Soybean mosaic virus* (SMV) in Ontario and Quebec

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Abstract: *Soybean mosaic virus* (SMV) can be present wherever soybean is grown, causing yield loss and seed quality deterioration. We have isolated SMV from eight soybean samples collected from fields in Ontario and Quebec, the two major soybean producing provinces in Canada. The coat protein region of these isolates was amplified, sequenced, and compared with 14 published sequences to determine the phylogenetic relationship among them. It was found that all eight isolates were most related to the SMV G2 group identified in the US. Further parsimony analysis of partial SMV sequences suggests that there are at least three SMV genotypes present in Ontario and two in Quebec. Three representative isolates were used for a pathogenicity test. As expected, all of them could infect a susceptible cultivar and a resistant cultivar carrying the resistance gene *Rsv3*, but could not infect resistant cultivars carrying the resistance genes *Rsv1*, *Rsv1-h*, or *Rsv4*.

Key words: Soybean mosaic virus, potyvirus, *Glycine max*, phylogenetic analysis.

Résumé : Le virus de la mosaïque du soja (SMV) peut se trouver partout où est cultivé le soya, causant des pertes de rendement ainsi qu'une détérioration de la qualité des semences. Nous avons isolé le SMV de huit échantillons de soja collectés dans des champs situés en Ontario et au Québec, les deux plus importantes provinces productrices de soja au Canada. La région de la protéine de coque de ces isolats a été amplifiée, séquencée et comparée à 14 séquences publiées afin d'en déterminer les relations phylogénétiques. Il s'est avéré que les huit isolats étaient principalement apparentés au groupe G2 du SMV identifié aux États-Unis. Une analyse additionnelle de parcimonie de séquences partielles de SMV suggère qu'il y a au moins trois génotypes de SMV en Ontario et deux au Québec. Trois isolats représentatifs ont été utilisés aux fins d'un test de pathogénicité. Comme on s'y attendait, tous pouvaient infecter un cultivar réceptif et un cultivar résistant porteur du gène de résistance *Rsv3*, mais ne pouvaient infecter les cultivars résistants porteurs des gènes *Rsv1*, *Rsv1-h* ou *Rsv4*.

Mots-clés : virus de la mosaïque du soja, potyvirus, *Glycine max*, analyse phylogénétique.

Introduction

Soybean mosaic virus (SMV), a monopartite, single-stranded positive sense RNA potyvirus, is the most common soybean virus worldwide. Following spread through seed, mechanical means, or by aphid vectors, the different SMV strains or mixtures of genotypes cause yield loss and seed quality deterioration in many soybean (*Glycine max* L. Merr.) production areas (Hill 2003). A study in China, for example, reported a 10% to 30% yield reduction due to SMV on soybean (Fu et al. 2006). Canada is one of the top seven soybean-producing countries in the world, with soybean

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Table 1. *Soybean mosaic virus* sequences used in this study.

Sample identifier	GenBank acc. No.	Origin	Reference
Novel isolates			
SMV_HRC1	EU931459	Canada (Ontario)	This study
SMV_HRC3	EU931458		This study
SMV_HRC4	EU931457		This study
SMV_HRC5	EU931456		This study
SMV_LON1	EU931460		This study
SMV_QC1	EU931453	Canada (Quebec)	This study
SMV_QC2	EU931454		This study
SMV_QC3	EU931455		This study
Reference contig sequences			
SMV_Contig 1	9 member sequences*	USA (South Carolina, Missouri, and Iowa)	Strömvik et al. (2006)
SMV_Contig 2	29 member sequences*	USA (South Carolina, Missouri, Illinois, and Iowa)	Strömvik et al. (2006)
SMV_Contig 3	35 member sequences*	USA (South Carolina, Missouri, and Illinois)	Strömvik et al. (2006)
SMV_Contig 4	38 member sequences*	USA (South Carolina)	Strömvik et al. (2006)
Reference genomes			
N	NC_002634 (D00507)	USA	Eggenberger et al. (1989)
G2	S42280	USA	Jayaram et al. (1992)
G5	AY294044	South Korea	Lim et al. (2003)
G7	AY216010	USA	Hajimorad et al. (2003)
G7*	AF241739	USA	Jayaram et al. (1992)
G7d	AY216987	USA	Hajimorad et al. (2003)
G7H	AY294045	South Korea	Lim et al. (2003)
Aa	AB100442	Japan	Yamagishi et al. (2003)
Aa15-M2	AB100443	Japan	Yamagishi et al. (2003)
Severe	AJ312439	China	Chen et al. (2004)

*The list of accession numbers for these contigs will be available from http://soyxpress.agrenv.mcgill.ca/ (Cheng and Strömvik 2008).

primarily grown in Ontario and Quebec (United States Department of Agriculture 2004). SMV was previously found in Ontario (Tu 1986). More recently, Gagarinova et al. (2008) isolated and characterized an SMV resistance-breaking isolate and its parental isolate from an experimental field in London, Ontario. However, SMV has not previously been reported in Quebec, and the phylogenetic relationship between isolates from Ontario and Quebec and between Canadian SMV isolates and the US isolates has not been investigated.

In this study, we have collected eight SMV isolates from Ontario and Quebec, cloned and sequenced the coat protein regions, and compared these sequences with those of known strains. Phylogenetic analysis suggests that different SMV isolates are present in these two provinces but that they all belong to the G2 group, a clade of SMV genotypes that is widespread in the US. Further pathogenicity tests confirm that these Canadian isolates are members of the G2 pathotype.

Materials and methods

SMV detection, RNA purification, reverse transcription polymerase chain reaction (RT-PCR), and sequencing

Soybean leaf samples showing typical SMV symptoms, i.e., mosaic, wrinkled, or puckered leaves, were collected from different fields in Harrow and London, Ontario, and in Sainte-Anne-de-Bellevue, Quebec, Canada. Double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) were carried out on the initial leaf samples from the field using the PathoScreen Kit (Agdia, Elkhart, Indiana) following the manufacturer's instructions. The field samples were used to inoculate soybean 'OAC Bayfield', a susceptible cultivar, grown at ambient humidity in a growth chamber with a 16 h photoperiod (450 $\mu mol \cdot m^{-2} \cdot s-1$) and a 25 °C day / 20 °C night temperature. The plants were placed in the dark for 24 h before inoculation. The inoculum was prepared by homogenizing 0.5 g of symptomatic soybean leaves (leaves from each field plant were used separately) with 1.2 mL of inoculation buffer (0.5 mol/L potassium phosphate buffer, ph 7.5, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), kept at 4 °C). The inoculum from each of the symptomatic field plants was applied to the first trifoliate leaves of each of four plants in the growth chamber. Twenty days post-inoculation, total RNA was isolated from systemically infected trifoliate leaves using the RNeasy Plant Mini Kit according to the manufacturer's protocol (Qiagen Science, Maryland). The RNA samples were reverse transcribed into cDNA using the Omniscript Reverse Transcription Kit (Qiagen Science) with an SMV reverse primer (5′-GATTCACATCCCTTGCAGT-3′). Standard PCR was performed to amplify cDNA derived from the RNA samples. The SMV reverse primer and an SMV forward primer (5′-TGATTGAGGCATGGGGATAC-3′) allowed specific amplification of a 1071 bp fragment encoding the SMV coat protein and 3′ untranslational region (8560–9631 in the SMV genome). The PCR products were gel-purified using the QiaQuick Gel Extraction Kit (Qiagen Science)

Table 2. Percent sequence identity among the analyzed coat protein region of *Soybean mosaic virus* (SMV) isolates from Quebec and

	SMV_ HRC1	SMV_ HRC3	SMV_ Contig 2	SMV_ LON1	SMV_ QC2	SMV_ QC3	G2	N	SMV_ QC1	SMV_ HRC4
SMV_HRC3	100.00									
SMV_Contig 2	99.56	99.6								
SMV_LON1	99.45	99.6	99.41							
SMV_QC2	99.23	99.3	99.12	99.78						
SMV_QC3	99.00	99.1	98.97	99.56	99.34					
G2	99.23	99.2	99.27	99.34	99.11	98.89				
N	99.23	99.2	99.26	99.56	99.34	99.11	99.34			
SMV_QC1	99.45	99.5	99.26	99.56	99.34	99.14	99.34	99.34		
SMV_HRC4	99.11	99.1	99.12	99.45	99.23	99.00	99.00	99.23	99.23	
SMV_HRC5	99.11	99.1	99.12	99.45	99.23	99.00	99.00	99.23	99.23	100.00
SMV_Contig 1	99.34	99.3	99.27	99.45	99.23	99.00	99.25	99.23	99.45	99.34
G7H	97.45	97.5	97.35	97.34	97.12	97.12	97.12	97.34	97.34	97.23
G5	97.56	97.6	97.35	97.45	97.23	97.23	97.23	97.23	97.45	97.34
SMV_Contig 3	96.23	96.2	96.32	96.34	96.11	95.89	95.89	96.34	96.11	96.00
Aa15_M12	95.24	95.3	95.45	95.13	94.91	94.91	94.68	94.91	94.91	94.80
Aa	95.24	95.3	95.45	95.13	94.91	94.91	94.68	94.91	94.91	94.80
G7d	95.02	95	94.72	94.91	94.68	94.68	94.91	94.91	94.68	94.80
G7	95.02	95	94.86	94.91	94.68	94.68	94.91	94.91	94.68	94.80
G7*	96.12	96.1	96.03	96.01	95.79	95.57	96.24	96.24	95.79	95.68
SMV_Contig 4	92.73	92.7	92.23	92.36	92.11	91.86	92.23	92.11	92.23	92.36
Severe	91.14	91.1	90.33	90.81	90.59	90.37	90.59	90.59	90.81	90.92

and directly subjected to sequencing in both directions at the McGill University and Génome Quebec Innovation Centre. To verify that they were virus coat proteins, the resulting SMV sequences were used as query sequences with BLASTX (Altschul et al. 1997) against the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/). The nucleotide sequences will appear in GenBank under the following accession numbers: EU931453–EU931460.

Phylogenetic analyses of the eight novel isolates and 14 previously known SMV isolates

The sequence chromatograms were trimmed and edited using the 4Peaks program, v. 1.7 (http://mekentosj.com/4peaks/). The names and origins of the new isolates, contig sequences, and publicly available reference genome sequences used in the analysis are shown in Table 1. The sequences were aligned using ClustalX (Thompson et al. 1997), and the alignment was subsequently refined by keeping only the sequence regions that were common to all the aligned sequences. Parsimony analysis was performed with PAUP* v. 4.0b10 for Macintosh (Swofford 2002) as described in Strömvik et al. (2006). The following nucleotide bases (characters), positioned with respect to the reference genomes, were included in the phylogenetic analysis and distance calculation: 8347–9249 for G2, G7*, and N; and 8350–9252 for G5, G7, G7d, G7H, Aa, Aa15-M2, and Severe strain (designated as the out-group taxon). A preliminary analysis generated an initial series of trees by keeping a maximum of 1 tree per step of stepwise addition, using HRC1 as the reference. These initial trees then served as starting trees for a heuristic search with the tree-bisection-reconnection (TBR) branch-swapping algorithm. Support values were calculated with 1000 full heuristic bootstrap replicates (Felsenstein 1985). Sequence identities were calculated with PAUP, for the same regions as those used for phylogenetic analysis.

Pathogenicity test of three representative isolates from Ontario and Quebec

'Williams 82', a susceptible soybean cultivar, and five resistant cultivars or accessions PI96983 (*Rsv1*), L78-379 (*Rsv1*), L29 (*Rsv3*), 'Suweon97' (*Rsv1-h*), and V94-5152 (*Rsv4*), containing an SMV resistance gene (indicated in italic) (Kiihl and Hartwig 1979; Buss et al. 1997, 1999; Chen et al. 2002), were grown in a growth chamber as described above. These cultivars were mechanically inoculated with three representative SMV isolates (SMV_HRC1, SMV_LON1, and SMV_QC1). The responses of these cultivars to the infection of these three SMV isolates were examined as described (Gagarinova et al. 2008).

Results and discussion

In this study, we collected symptomatic soybean leaf samples from fields in Ontario and Quebec, detected the presence of SMV by DAS-ELISA, and amplified partial SMV genome using RT-PCR, followed by sequencing and sequence analysis. Using the ELISA kit, samples SMV_LON1 and SMV_HRC1, 2, 3, 4, and 5 from Ontario tested positive for SMV, whereas the Quebec samples (SMV_QC1, 2, and 3) were negative. RT-PCR was further used to detect SMV in all samples. A DNA fragment corresponding to the predicted size for the CP cistron was evident in all the samples from Ontario and Quebec, except for SMV_HRC2, possibly because of SMV RNA degradation during the shipment of

Ontario, reference sequences from GenBank, and contig sequences of SMV.

SMV_ HRC5	SMV_ Contig 1	G7H	G5	SMV_ Contig 3	Aa15_ M12	Aa	G7d	G7	G7*	SMV_ Contig 4
99.34										
97.23	97.45									
97.34	97.56	99.89								
96.00	96.23	95.01	94.89							
94.80	95.24	94.91	94.80	93.56						
94.80	95.24	94.91	94.80	93.56	100.00					
94.80	94.80	94.68	94.57	93.23	96.57	96.57				
94.80	94.80	94.68	94.57	93.23	96.79	96.79	99.78			
95.68	95.68	95.13	95.02	94.11	96.12	96.12	98.23	98.45		
92.36	92.36	92.48	92.48	91.35	92.85	92.85	92.11	92.23	92.23	
90.92	91.03	90.59	90.70	90.01	90.81	90.81	90.00	90.26	90.26	96.30

this sample, which was therefore excluded from the rest of the study. The RT-PCR amplified DNA fragment was gel-purified and directly sequenced. BLASTX analysis against the NCBI database indicated that all DNA samples were derived from the SMV genome. It is possible that the negative ELISA results with the Quebec samples was because the SMV titres were too low for this technique. Table 1 provides the names, origin, and reference for the novel sequences obtained in this study and the SMV sequences reported previously.

In a previous study, the phylogenetic relationship was determined based on 953 bp of the coat protein region from US-derived SMV sequences present in four contigs (SMV_Contig1, 2, 3, and 4) assembled from EST analysis and the corresponding region of 10 completely sequenced SMV genomes (G2, G5, G7, G7*, G7d, G7H, N, Aa, Aa15-M2, and Severe strain; Table 1.) (Strömvik et al. 2006). In this study, we used 904 bp of the coat protein region to determine the phylogenetic relationship between the eight SMV isolates from Ontario and Quebec obtained in this study and the 14 US strains and isolates published previously. Because the SMV Severe strain was found by parsimony analysis to be the most distant, it was designated the out-group. Table 2 shows the percent identity among all 22 sequences analyzed.

The PAUP* (Swofford 2002) parsimony analysis retrieved only the most parsimonious tree (length = 223, consistency index = 0.767, retention index = 0.836), which is shown in Figure 1. Among the 904 nucleotides considered, a total of 113 were parsimony-informative characters and 54 were variable.

All the Canadian isolates, SMV_HRC1, SMV_HRC3, SMV_HRC4, SMV_HRC5 SMV_LON1, SMV_QC1, SMV_QC2, and SMV_QC3, sorted in the largest clade (79% bootstrap) together with the SMV_Contig 2, SMV_Contig 1 sequences, and the G2 and N genotypes. Within this clade, the SMV_HRC4 and SMV_HRC5 formed a consistent clade with 95% bootstrap and 99% nucleotide identity and SMV_LON1, SMV_QC2, and SMV_QC3 formed a low-consistency clade with a bootstrap of 62% and >99% nucleotide identity. All sequences in this large clade were very similar among isolates, with nucleotide identities typically over 99%. The London isolate (SMV_LON1) was more similar to two Quebec isolates (SMV_QC2 and SMV_QC3) than to the Harrow isolates. The grouping in the rest of the tree was consistent with the previous study (Strömvik et al. 2006).

Three SMV isolates (SMV_LON1, SMV_HRC1, and SMV_QC1) from London, Harrow (Ontario), and Sainte-Anne-de-Bellevue (Quebec), respectively, were used for a pathogenicity test. As shown in Table 3, all three isolates could infect a susceptible cultivar and a resistant cultivar carrying resistant gene *Rsv3*, but could not infect resistant cultivars carrying resistant genes *Rsv1*, *Rsv1-h*, or *Rsv4*. These results suggest that these three representative isolates belong to the G2 pathotype (Gagarinova et al. 2008), consistent with its grouping in the parsimony analysis.

In conclusion, all SMV isolates from Quebec and Ontario used in this study were present in the same main clade and were similar to SMV_Contig 1 and SMV_Contig 2 (Strömvik et al. 2006) and the G2 and N SMV genotypes. In the previous study, SMV_Contig 1 and SMV_Contig 2 were shown to be the more heterologous contigs, and consisted of SMV sequences from plants that were grown in different regions of the USA. This is in contrast to the more distant Severe strain and SMV_Contig 4, which were found

Fig. 1. Phylogeny of SMV isolates. All eight isolates from Ontario (SMV_LON1, SMV_HRC1, 3, 4, 5) and Quebec (SMV_QC1, 2, 3) are in the same clade as the G2 strain, which is a known and widespread strain in the US. The bootstrap values are given above the branches.

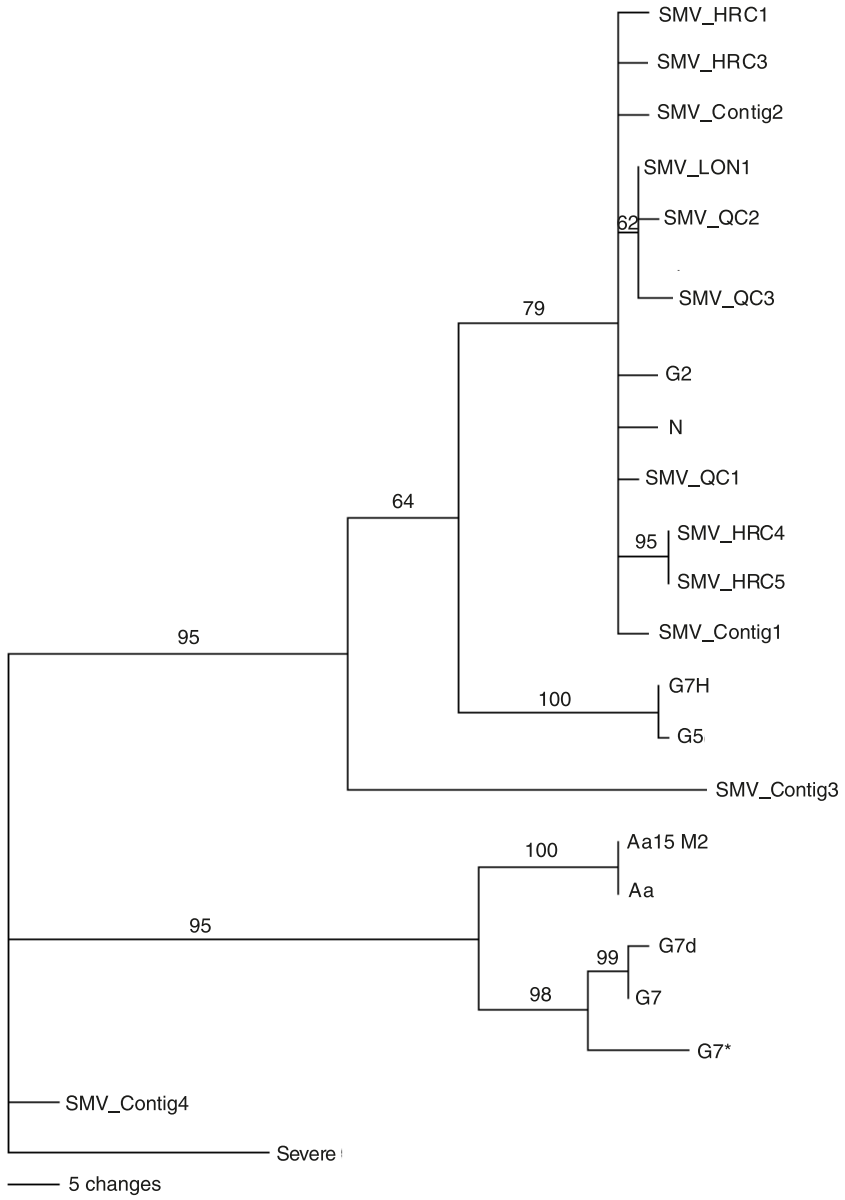

Table 3. Pathogenicity of Canadian SMV isolates in different soybean cultivars.

			Response to different isolates at 21 days post inoculation		
Cultivars or accessions	Resistance allele	Description	London	Harrow	Quebec
'Williams 82'	none	Susceptible indicator (Jayaram et al. 1992)	Severe mosaic, some crinkling	Severe mosaic, some crinkling	Severe mosaic, some crinkling
PI 96983	*Rsv1*	Resistant to G1–G6, but not to G7 (Yu et al. 1994; Gunduz et al. 2002)	Not infected	Not infected	Not infected
L78-379	*Rsv1*	Williams 82 isoline with *Rsv1* from PI 96983 (Gunduz et al. 2002)	Not infected	Not infected	Not infected
L29	*Rsv3*	Resistant to G5–G7, but susceptible to G1-G4 (Gunduz et al. 2002)	Severe mosaic, severe crinkling	Severe mosaic, severe crinkling	Severe mosaic, severe crinkling
'Suweon 97'	*Rsv1-h*	Resistant to all characterized SMV strains (G1–G7) (Chen et al. 2002)	Not infected	Not infected	Not infected
V94-5152	*Rsv4*	Resistant to all characterized SMV strains (G1–G7) (Gunduz et al. 2002)	Not infected	Not infected	Not infected

in the Southern states only (Strömvik et al. 2006). These data suggest that the G2 strain is highly endemic in the US, and thus it is not surprising that all the SMV isolates identified in Canada also belong to the G2 strain. Since SMV is highly transmissible mechanically, by seed or by aphids, the quality of seed and the choice of virus resistant soybean cultivars must receive careful attention to reduce future problems associated with SMV in these areas.

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