SHORT COMMUNICATION

IDENTIFICATION OF *SOYBEAN MOSAIC VIRUS* IN POLAND

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SUMMARY

Soybean mosaic virus (SMV), the causal agent of a commonly occurring disease of soybean (*Glycine max*), is seed-borne and readily transmitted mechanically. Soybean plants and seeds in Poland were surveyed by ELISA to evaluate the occurence of SMV and characterize its isolates by RT-PCR using primers designed in the P1, HC-Pro and coat protein (CP) genes followed by sequencing. Two isolates designed A and M differed biologically with SMV-A infecting soybean plants without apparent symptoms and SMV-M causing foliar rugosity and curling, and stunted growth. The CP gene of the two isolates had 92% and 99% identity at the nucleotide and amino acid level, respectively. Phylogenetic analyses showed that the two Polish isolates grouped in two distinct clusters. This is the first report on SMV identification in Poland.

Key words: SMV, detection, RT-PCR, soybean, seeds.

Soybean mosaic virus (SMV) is a seed-transmitted pathogen of soybean. As other pathogens transmitted via seeds, it can be widely distributed, expanding to all regions where its host is cultivated. The main areas of soybean production are in North America (USA), Asia (Japan, China) and in Africa (Goodman, 1996). Investigations on SMV in Europe were initiated in the 1980s and an ELISA protocol was developed for SMV detection in seed stocks using the American type of SMV strains (Maury *et al*., 1983, 1985). Sherepitko *et al.* (2011) reported the isolation of SMV and partial sequencing of strains from Ukraine. This was the first report of the virus in Europe. In Poland, soybean is not a major crop but three Polish cultivars (Nawiko, Aldana and Augusta) suitable for local production are available although cultivars of foreign origin are promising. Soybean hosts over 67 viruses, among which 27 are threatening production (Saghai Maroof *et al*., 2008). SMV (family *Potyviridae*) is commonly believed to be the most detrimental virus of soybean (Cui *et al*., 2011). This investigation was undertaken to survey soybean seed stocks in Poland for the presence of SMV and to evaluate the risk of introducing new strains with the adoption of foreign lines and/or cultivars. Soybean seeds of cvs Aldana, Augusta and Sultana and fields plants of cvs Aldana, Anushka, Augusta, Lissabon, Mavka and Merlin were surveyed for SMV in July 2013 and 2014. Seeds of cvs Aldana and Augusta were obtained from the Experimental Station of the Institute of Plant Protection-National Research Institute in Winnagóra, and seeds of cv. Sultana were kindly provided by Plant Breeding Station DANKO in Szelejewo.

Plant samples were randomly collected in three locations, two in the Wielkopolska region and one near the south west border of Poland, because no disease symptoms were noticed. Preliminary diagnostics for SMV was performed by ELISA (Clark and Adams, 1977) using commercial antibodies (Loewe, Germany). An indirect procedure was used to detect the virus in young seedlings grown from seeds (Jeżewska, 2006). Results showed the presence of SMV in 69 out of 728 seedlings tested (Table 1). The highest percentage of infected seeds was recorded in cvs Sultana (9.5%) and Aldana (7.1%). The OD values for infected plants did not exceed 0.3, with an average of about 0.1 in comparison to 0.005-0.01 for healthy plants, suggesting a low virus titer. SMV was detected in only three out of 262 plants of cvs. Anushka and Merlin. The OD value for cv. Anushka plants was as low as 0.07. However, in the case of wo plants of cv. Merlin, OD values ranged from 0.4 to 0.8.

Table 1. Detection of *Soybean mosaic virus* in soybean seeds and field plants.

Cultivar	Number of seeds		Number of plants	
	Tested	SMV infected	Tested	SMV infected
Aldana	380	27	45	0
Anushka	0	0	20	1
Augusta	96	0	40	0
Lissabon	0	0	45	0
Mavka	0	0	40	0
Merlin	0	0	72	2
Sultana	348	42	0	0

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Table 2. Primers used in the study**.**

Primer	Primer sequence 5'- 3'	Position *	Amplicon size (bp)
SMV-P1-F	GCATGGCGATTTCTGTGCC	154-172	756
SMV-P1-R	CAAACAGTAAACCACTATCTC	933-913	
SMV-Hc-F	GGCAGCGGCAATAAGCCAATC	1178-1198	1030
SMV-Hc-R	CATCTAACATTGTCGGCCAC	2208-2189	
SMV3-F	GTGAGCTACAAAGATATCTTG	8464-8484	913
SMV1-R	TCGGTCTGACCCTGTTCTTC	9374-9355	

* The primers' position according to SMV-HZ (Ac. No. AJ312439).

Two Polish SMV isolates, designed A and M, were selected for characterization. SMV-A was isolated from a plant of cv. Aldana and SMV-M from a plant of cv. Merlin. The virus was maintained on soybean plants by mechanical inoculation. All test plants were grown in a greenhouse. Soybean plants infected with SMV-A were symptomless. The isolate was also difficult to transmit mechanically in contrast to SMV-M. Symptoms observed in the cv. Merlin plant naturally infected with SMV-M were very mild and difficult to distinguish among other field-grown plants. However, after mechanical transmission, infected plants exhibited rugosity, leaf curling, stunting and a strong decrease in seed production, as described by Goodman (1996). Symptoms appeared about 5-6 days post inoculation. Among the seven plant species (*Antirrhinum majus*, *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana tabacum* cv. Xanthi, *Phaseolus vulgaris*, *Pisum sativum* and *Vigna unguiculata)* inoculated mechanically with SMV, only *C. quinoa* showed symptoms that consisted of local tiny chlorotic lesions *ca.* 1-2 mm in diameter.

The presence of filamentous, flexuous virus particles about 700×15 nm in size was confirmed in infected soybean plants by electron microscopy observations. The samples were prepared by grinding small pieces of leaf tissues in water, staining with 2% potassium phosphotungstic acid (pH 7.2) and examined with a Hitachi HT7700 transmission electron microscope at an acceleration voltage of 80 kV. Microscopic observations also revealed more virus particles (20-25 per grid) for plant extracts infected with SMV-M compared to SMV-A (3-10 per grid).

Total RNA was extracted from 100 mg of infected soybean plant leaves using the NucleoSpin RNA Plant kit (Macherey-Nagel, Germany) according to manufacturer's instructions. Isolated RNA was amplified in RT-PCR with primers designed in the P1 (protease), HC-Pro (helper component-protease), and coat protein (CP) coding regions using the Primer3 software (http://frodo.wi.mit.edu) (Rosen and Skaletski, 2000) based on conserved regions after alignment of full-length SMV sequences retrieved from GenBank (Table 2). First-strand cDNAs were synthesized using SuperScript Reverse Transcriptase (Invitrogen, USA) with primers SMV-P1-R, SMV-HC-R and SMV1-R, and PCR was carried out in a 10 µl final reaction volume using 1 µl cDNA and the AccuPrime Taq DNA Polymerase System (Invitrogen, USA) reaction mix with appropriate primer pairs. Amplification was performed as follows: 94°C for 2 min, 40 cycles of 94°C for 30 sec, 55°C for 30 sec, 68°C for 1 min and a final extension at 68°C for 5 min. PCR products were separated by electrophoresis on 1% agarose gel with the Midori Green DNA Stain (Nippon Genetics Europe GmbH) and visualized under UV. DNA amplicons of the expected size were obtained for the P1 (756 bp), HC-Pro (1030 bp) and CP (913 bp) genes. The PCR products were eluted from gels using Wizard SV Gel and PCR Clean-UpSystem (Promega, USA). Each fragment was ligated into the pGEM T-Easy system cloning vector (Promega, USA). Plasmid DNA was isolated using NucleoSpin Plasmid (NoLid) (Machery-Nagel, Germany). Subsequently, three recombinant clones of each fragment were sequenced by Genomed (Poland) on both strands using M13-F and M13-R primers. Nucleotide sequences were analyzed using the BlastN program, compiled and edited in the BioEdit software (Hall, 1999) and deposited in GenBank with the following accession numbers: KM886427 (partial P1 fragment of SMV-M), KM886428 (partial HC-Pro fragment of the SMV-M), KM886429 (full-length CP gene of SMV-M) and KM886430 (full-length CP gene of SMV-A).

De novo designated primers (SMV-P1-F/SMV-P1-R, SMV-Hc-F/SMV-Hc-R and SMV3-F/ SMV1-R) generated the expected products of 756 bp, 1030 bp and 913 bp, respectively. A comparison of the CP gene of both isolates revealed a 92% nucleotide sequence identity and a 99% amino acid sequence identity. Amino acids alignment of the CP gene sequence showed that SMV-A was most similar to American isolates G3, TNP (99.3%), KY, 413 and G1 (99%), and to Iranian isolates Ar33, Go11 and Lo3 (99%). The same analysis demonstrated an identical amino acid sequence identity of SMV-M with the Iranian isolates Ar33, Go11, and Lo3. In the N-terminus of the P1 gene an identical amino acid composition was found for SMV-M and isolate UA1Gr from Ukraine. Analysis of a partial fragment of the HC-Pro gene of SMV-M indicated 100% identity at the amino acid level with Canadian isolates L, L-RB, NP-C-L, NP-L and Rsv4-RB3. The coding sequence of protein P1 was more variable than the other HC-Pro and CP regions. These results are consistent with those from the literature (Saghai Maroof *et al*., 2008; Seo *et al*., 2009b).

Phylogenetic analyses of the nucleotide and amino acid sequences were conducted for Polish SMV isolates A and M, and 41 previously characterized isolates for which the CP gene sequence was determined (Table 3). Multiple

Table 3. SMV isolates analyzed in this study.

SMV isolate	Accession No.	Geographic origin	Original host	Reference
G1	FJ640977	USA	*Glycine max*	Seo *et al.* (2009b)
G2	S42280	USA	*Glycine max*	Jayaram *et al.* (1992)
G3	FJ640978	USA	*Glycine max*	Seo *et al.* (2009b)
G4	FJ640979	Korea	*Glycine max*	Seo *et al.* (2009b)
G5	AY294044	Korea	*Glycine max*	Lim *et al.* (2003)
G5H	FJ807701	Korea	*Glycine max*	Seo *et al.* (2009a)
G6	FJ640980	Korea	*Glycine max*	Seo *et al.* (2009b)
G6H	FJ640981	Korea	*Glycine max*	Seo *et al.* (2009b)
G7	AY216010	USA	*Glycine max*	Hajimorad *et al.* (2003)
G7a	FJ640982	USA	*Glycine max*	Seo *et al.* (2009b)
KY	HQ845736	USA	*Glycine max*	Khatabi *et al.* (2012)
N	D00507	USA	-	Eggenberger *et al.* (1989)
TNP	HQ845735	USA	*Glycine max*	Khatabi *et al.* (2012)
413	GU015011	USA	*Glycine max*	Domier *et al.* (2011)
VA2	AF200582	USA	-	Unpublished
WS84	FJ640956	Korea	*Glycine soja*	Seo *et al.* (2009b)
WS155	FJ640970	Korea	*Glycine soja*	Seo *et al.* (2009b)
WS162	FJ640973	Korea	*Glycine soja*	Seo *et al.* (2009b)
Aa	AB100442	Japan	-	Unpublished
HZ	AJ312439	China	*Glycine max*	Chen *et al.* (2004)
HH5	AJ310200	China	*Glycine max*	Chen *et al.* (2004)
SC3	JF833013	China	*Glycine max*	Unpublished
Sc6	HM590054	China	*Glycine max*	Yang *et al.*(2011)
SX	KC845321	China	*Glycine max*	Unpublished
L	EU871724	Canada	*Glycine max*	Gagarinova *et al.* (2008)
L-RB	EU871725	Canada	*Glycine max*	Gagarinova *et al.* (2008)
NP-C-L	HQ166265	Canada	*Glycine max*	Chowda-Reddy *et al.* (2011)
NP-L	HQ166266	Canada	*Glycine max*	Chowda-Reddy *et al.* (2011)
Rsv4-RB3	JN416770	Canada	*Glycine max*	Chowda-Reddy *et al.* (2011)
Ar13	KF135488	Iran	*Glycine max*	Ahangaran *et al.* (2013)
Ar15	KF135467	Iran	*Glycine max*	Ahangaran *et al.* (2013)
Ar16	KF135468	Iran	*Glycine max*	Ahangaran *et al.* (2013)
Ar18	KF135469	Iran	*Glycine max*	Ahangaran *et al.* (2013)
Ar33	KF135489	Iran	*Glycine max*	Ahangaran *et al.* (2013)
Go6	KF135471	Iran	*Glycine max*	Ahangaran *et al.* (2013)
Go11	KF135491	Iran	*Glycine max*	Ahangaran *et al.* (2013)
Go34	KF135478	Iran	*Glycine max*	Ahangaran *et al.* (2013)
Lo3	KF135490	Iran	*Glycine max*	Ahangaran *et al.* (2013)
MA43	KF135486	Iran	*Glycine max*	Ahangaran *et al.* (2013)
MA50	KF135487	Iran	*Glycine max*	Ahangaran *et al.* (2013)
UA1Gr	JF803911	Ukraine	*Glycine max*	Sherepitko *et al.* (2011)
UA1Gr	JF431105	Ukraine	*Glycine max*	Sherepitko *et al.* (2011)
A	KM886930	Poland	*Glycine max*	This study
M	KM886927	Poland	*Glycine max*	This study
M	KM886928	Poland	*Glycine max*	This study
M	KM886929	Poland	*Glycine max*	This study
WMV-Fr	AY437609	France	*Zucchini squash*	Desbiez and Lecoq (2004)

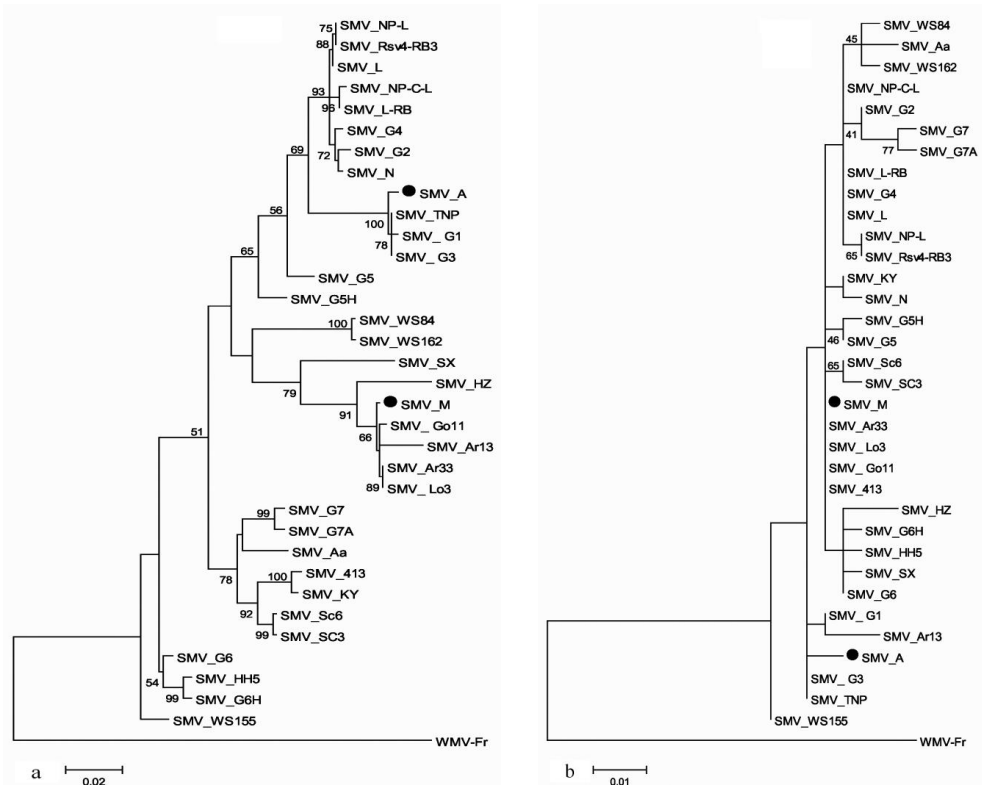

Fig. 1. Maximum-likelihood (ML) trees resulting from nucleotide sequences (a) or amino acid sequences (b) of the SMV-CP gene from different isolates (names and accession numbers are given in Table 3). The numbers of each major node indicate bootstrapping values (shown only when >50%) out of 1000 replicates. The scale bar shows the number of character substitutions per base to indicate total nucleotide diversity amongst taxa. *Watermelon mosaic virus* was used as an outgroup.

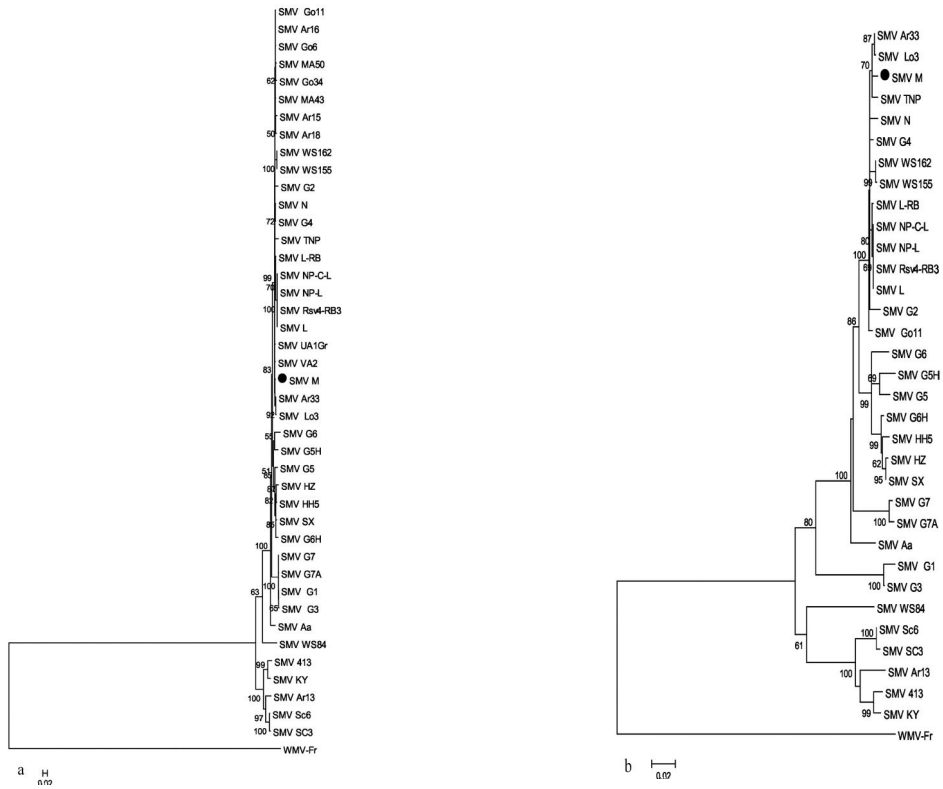

Fig. 2. Maximum-likelihood (ML) trees resulting from nucleotide sequences of SMV-P1 (a) and SMV-HC-Pro (b) gene fragments from different isolates (names and accession numbers are given in Table 3). The numbers of each major node indicate bootstrapping values (shown only when >50%) out of 1000 replicates. The scale bar shows the number of character substitutions per base to indicate total nucleotide diversity amongst taxa. *Watermelon mosaic virus* was used as an outgroup.

alignments were performed using the ClustalW software. The phylogenetic relationships of the SMV sequences were analyzed by the Maximum Likelihood algorithm (ML) implemented in the MEGA 6.1 program. Bootstrap values were calculated using 1000 random replications. Phylogenetic trees were visualized using TreeExplorer implemented in the MEGA 6.1 program.

Phylogenetic relationships based on the CP gene revealed that Polish isolates belong to two distinct clusters. SMV-A grouped with the American isolates G1, G3 and TNP, whereas SMV-M grouped with Iranian isolates Ar33, Go11and Lo3 (Fig. 1a, b). Phylogenetic trees of the P1 and HC-Pro gene fragments confirmed a close relationship of Polish SMV-M with Iranian isolates Ar33 and Lo3 (Fig. 2a, b). These results indicated at least two independent introduction events of SMV to Poland, most probably through infected soybean seeds. Our phylogenetic analyses did not show any clear relationship between genetic variability and the geographic origin of SMV isolates, confirming previous findings (Sherepitko *et al*., 2011; Seo *et al*., 2009b).

This is the first report on SMV occurrence in Poland. Our findings show that commercial soybean seeds are not SMV-free, in spite of the fact that the infections were asymptomatic. Such information should be taking into account in view of the increased production of soybean in Europe.

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