Detection of RNA1 and RNA2 of *Soil-borne wheat mosaic virus* in winter wheat grown from infected seeds

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Abstract: A Polish isolate of *Soil-borne wheat mosaic virus* (SBWMV-Pol1) was characterized by limited pathogenicity and a low concentration of virus particles in infected plant tissues. The aim of this research was to consider the possibility of seed-transmission dissemination of the virus. Seeds of winter wheat cv. Muszelka served as material for the studies. Two methods were involved in the diagnostics of seedlings grown from potentially infected seeds: enzyme-linked immunosorbent assay (ELISA), as the screening assay and immuno-capture-reverse transcription-polymerase chain reaction (IC-RT-PCR) for molecular confirmation of the infection. RNA1 and RNA2 of SBWMV-Pol1 were detected in 6 out of 1,410 plants submitted to diagnostic procedures. The possibility of seed transmission of SBWMV-Pol1 was discussed.

Key words: epidemiology, *Soil-borne wheat mosaic virus*, wheat

Introduction

Soil-borne wheat mosaic virus (SBWMV), belonging to the genus *Furovirus*, vectored by soil-inhabiting plasmodiophorid *Polymyxa graminis* Led., was first found by McKinney (1923) to be the causal agent of mosaic symptoms in winter wheat in the United States. In Europe it was identified about 40 years later (Canova 1964). Subsequent investigations revealed the occurrence of SBWMV in several European countries (Kühne 2009). The pathogen was reported to cause significant yield losses in wheat crops (Palmer and Brakke 1975; Vallega and Rubies-Autonell 1985; Drumm-Myers *et al.* 1993; Clover *et al.* 2001).

Further studies of the collected furovirus isolates, originating from various geographic regions, revealed differences among viruses in the genus *Furovirus* (Shirako *et al.* 2000). Later results led to the verification of the taxonomic classification. The former SBWMV species was divided into three related new species, differing in some biological, serological and molecular properties. Thus the name *Soil-borne wheat mosaic virus* was reserved for American isolates. European isolates were grouped into a new species named *Soil-borne cereal mosaic virus* (SBCMV) and the virus in China was designated *Chinese wheat mosaic virus* (CWMV) (Diao *et al.* 1999; Koenig and Huth 2000; Adams 2009). Such differentiation created confusion in Europe since all previous papers referring to SBWMV in fact concerned SBCMV. In 2003 Koenig and Huth published a communication on the identification of true SBWMV in Germany, according to the existing definition of the virus species.

In Poland the occurrence of SBWMV was reported by Jeżewska (1994) but later it was identified to actually be SB-CMV (Koenig and Huth 2000; Jeżewska and Trzmiel 2009). From 2005 to 2010 routine monitoring of winter wheat and winter triticale crops was conducted (Jeżewska *et al.* 2010; Jeżewska and Trzmiel 2010). SBCMV was found in different regions of Poland. During these studies another furovirus, SBWMV, was identified in triticale plants. The isolate, named SBWMV-Pol1, was characterized by generally low virulence associated with a limited concentration of virus particles in plant tissues (Trzmiel *et al.* 2012).

SBWMV-Pol1 underwent molecular characterization (Trzmiel *et al.*, unpublished data). Furthermore, specific biological properties prompted us to analyze the possibility of its seed transmission. The objective of our investigation was to detect viral RNA1 and RNA2 in plants grown from infected seeds.

Materials and Methods

Soil-borne wheat mosaic virus isolate-Pol1, collected from Szelejewo (Southern Greater Poland), was described by Trzmiel *et al.* (2012). The virus was propagated in wheat plants cv. Muszelka by infested soil-transmission and maintained in a climatic chamber at a controlled temperature of 17°C (Vaïanopoulos *et al.* 2005). Two German isolates, SBWMV-Heddesheim (from Heddesheim-Baden-Württemberg) and SBWMV-SH1-3 (from Cashagen-Schleswig-Holstein), obtained from the Julius Kühn Institut (Quedlinburg, Germany), served as reference isolates. Both German isolates were provided in the form of infested soils containing viruliferous spores of the vector with the viral strains, propagated in wheat plants cv.

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Muszelka and in triticale plants cv. Agostino by soil transmission and maintained in a climatic chamber at the temperature described above.

Cereal seeds were obtained from the Plant Breeding Station DANKO, Szelejewo.

SBWMV-Pol1 detection was carried out by two methods. Initial screening tests were performed by enzyme-linked immunosorbent assay (ELISA) (Clark and Adams 1977). Immunoglobulin, conjugate and positive controls were provided by Loewe Biochemica GmbH (Sauerlach, Germany). A sample was considered positive when its optical density (OD) value was at least three-times higher than the average OD of the negative control (healthy plants). Molecular confirmation of the positive results was performed by immuno-capture-reverse transcription-polymerase chain reaction (IC-RT-PCR). First, thin-wall polypropylene PCR tubes were coated with a 20 µl mixture containing 10-fold diluted monoclonal anti-SBWMV antibody with a commercial coating buffer (Loewe) and incubated for 1 h at 37°C. Then, tubes were washed three times in a commercial washing buffer (Loewe). Next, the plant tissue was homogenized using a mortar and pestle in a commercial conjugate buffer (Loewe). The coated reaction tubes were incubated with 50-µl plant sap for 1 h at 37°C to allow SBWMV particles to attach to the tube walls. Following washing in washing buffer, the PCR tubes were used for RT-PCR analysis. Assays were conducted using the OneStep RT-PCR Kit (Qiagen, Hilden, Germany). The reactions were performed in a final volume of 10 µl according to the manufacturer's instructions using primer pairs SWmpkr-F/SWmpkr-R amplifying movement protein (MP) fragment on RNA1 and SWdiag-F/SWdiag-R for the detection of the coat protein (CP) fragment on RNA2 of SBWMV (Table 1). Additional reactions were performed with SBWMVCPF/SBWMVCPR amplifying complete coding sequence of CP gene (695 bp) (Trzmiel *et al*. 2012).

The primers were designed using Primer3 software (http://frodo.wi.mit.edu/) (Rosen and Skaletski 2000) based on the full nucleotide sequence of RNA1 and RNA2 of American SBWMV isolate US-Nebraska wild-type (L0793 and L07938).

The cycling parameters were as follows: reverse transcription for 30 min at 50°C, initial PCR activation step for 15 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C and final extension for 10 min at 72°C. Polymerase chain reaction products were separated by 1% agarose gel electrophoresis and stained with Midori Green DNA Stain (NIPPON genetics Europe GmbH, Düren, Germany) for UV light visualization.

The PCR products of expected size were excised from the agarose gel, purified using Wizard®SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI, USA), ligated into the pGEM-T Easy Vector (Promega) and transformed into *Escherichia coli* TOP10 competent chemical cells (Thermo Fisher Scientific). Plasmid DNA was isolated using NucleoSpin® Plasmid (NoLid) (Macherey-Nagel) according to the manufacturer's instructions. Subsequently, at least three clones of each fragment were commercially sequenced by Genomed S.A. (Warsaw, Poland) in both directions with M13-F and M13-R primers. The nucleotide sequences were analyzed using Standard Nucleotide BLAST (BLASTN, http://blast.ncbi.nlm.nih.gov/Blast.cgi), compiled and edited in the BioEdit software (Hall 1999).

Winter wheat cv. Muszelka seeds collected from crops on land with plants infected with SBWMV-Pol1 served as material for seed transmission experiments.

Two indirect procedures were developed for the detection of SBWMV-Pol1 in seeds.

The first procedure consisted of sowing seeds into sterilized soil. They were kept under greenhouse conditions at about 24°C without any contact with any other potential sources of SBWMV infection. At the 3-leaf-stage the plants were diagnosed as described above. Only leaves were taken for diagnostics.

In the second procedure kernels of cv. Muszelka were soaked in distilled water on a thin layer of cotton in Petri dishes (20 cm in diameter) and left for 4 days for germination at room temperature. Before germination the seeds were washed with 0.026 M Na_3PO_4 for 10 min to remove any virus capsid from the surface of the seeds (Delfosse *et al*. 1999). Four-day-old whole seedlings (leaves and roots) were taken for diagnostics. Extracts of samples were divided into two parts, for ELISA and IC-RT-PCR (performed only for those indicated as positive in the first test).

Results and Discussion

Results of the detection of SBWMV-Pol1 in wheat cv. Muszelka seeds are summarized in Table 2.

A total of 1,410 plants grown from potentially infected seeds were diagnosed, 940 at 3-leaf-stage from a greenhouse and 470 as 4-day-old seedlings germinated in Petri dishes.

Table 1. Diagnostic primers designed for RNA1 and RNA2 of *Soil-borne wheat mosaic virus* (SBWMV)

Primer	Sequence 5'–3'	Position	Amplicon size [bp]
SWmpkr-F	CCTGTCACCACTTGAAAATGT	5976-5996	400
SWmpkr-R	GTTCTCTTTCATAACGTTAATCTGC	6375-6354	
Swdiag-F	CAATCCGCTAGAGGTGAAGG	631-650	229
Swdiag-R	CTCGAACCTTCCCATTTCAA	860-841	

Table 2. Detection of *Soil-borne wheat mosaic virus* (SBWMV-Pol1) in winter wheat cv. Muszelka plants grown from infected seeds (number of positive samples/number of assayed are given)

Length of storage time [months]	Leaves of 3-leaf-stage plants from greenhouse		4-day-old seedlings germinated in Petri dishes	
	ELISA	IC-RT-PCR	ELISA	IC-RT-PCR
< 6	70/640	5/52	4/278	1/4
> 6	38/300	0/10	10/192	0/2

ELISA – enzyme-linked immunosorbent assay; IC-RT-PCR – immuno-capture-reverse transcription-polymerase chain reaction

The first experiment included leaves of greenhouse grown plants at the 3-leaf-stage. In the ELISA test there were 108 (11.5%) positive results. However OD values indicated low virus concentration. The highest OD was 0.5 while OD for negative controls ranged from 0.001 to 0.09. This elevated rate of SBWMV-Pol1 infection was verified negatively by IC-RT-PCR. After molecular characterization five samples out of 52 were demonstrated to be infected with SBWMV-Pol1. RNA1 and RNA2 fragments, 400 bp (Fig. 1) and 229 bp (not shown), respectively, were visualized in an electrophoretic pattern.

Moreover, SBWMVCPF/SBWMVCPR in IC-RT-PCR generated an expected product of 695 bp, in three samples (Fig. 2).

All samples in which the virus infection was confirmed molecularly were shown to display OD values above 0.2. The products were cloned and sequenced. The nucleotide sequences of the longest amplicons were consistent with the coding sequence of the CP gene fragment of the Polish SBWMV-Pol1 isolate (JQ231227). Sequencing results of about 400 nucleotides of the next product indicated that they corresponded to nucleotides 391 to 711 of the MP SBWMV-Pol1 gene (KU193755) (Trzmiel *et al.* 2016, unpublished data) and confirmed viral specificity. All positive results were obtained for samples in which storage time did not exceed 6 months. Seeds stored for a longer time displayed a tendency to decrease the number of positive results in ELISA and a weak concentration of the virus. No specimen was confirmed molecularly.

The second experiment involved 4-day-old whole seedlings assayed directly from Petri dishes. Only one seedling out of 470 was found to be SBWMV-Pol1 infected by both methods. The number of positive results in ELISA was limited to 14 (3%) and the OD values did not exceed 0.3. No sample was found to be infected after 6 months of storage.

It appears that the most important result was the detection of both SBWMV-Pol1 RNAs in six plants grown from infected seeds. The identity of the amplified genome fragments was proven on the basis of nucleotide sequences of the amplicons.

It is interesting to note that the virus concentration declined during storage. Such an effect was already signaled earlier (Mink 1993).

The objective of the studies was to verify the capacity of SBWMV-Pol1 for seed transmission. The mild character of the virus isolate contrasted with data reported by other researchers (Lebas *et al.* 2009; Kastirr *et al.* 2012).
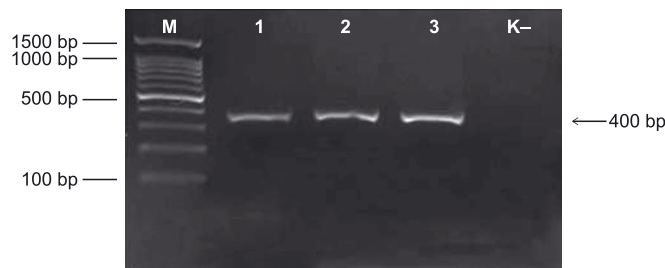

Fig. 1. Electrophoretic mobility of immuno-capture-reverse transcription-polymerase chain reaction (IC-RT-PCR) products with SWmpkr-F/SWmpkr-R on 1% agarose gel: M – marker DNA Nova 100 bp DNA ladder (Novazym); 1–3 – studied samples; K – negative control
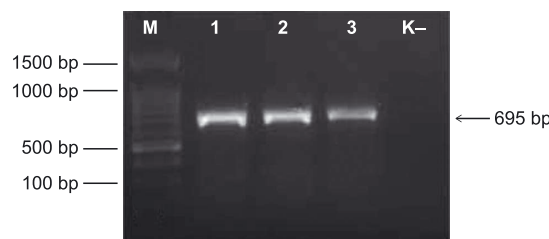

Fig. 2. Electrophoretic mobility of immuno-capture-reverse transcription-polymerase chain reaction (IC-RT-PCR) products with SBWMVCPF/SBWMVCPR on 1% agarose gel: M – marker DNA Nova 100 bp DNA ladder (Novazym); 1–3 – studied samples; K – negative control

This harmless nature of SBWMV-Pol1 was strictly connected with a low concentration of virus particles detected in plant tissues. Hypothetically SBWMV-Pol1 could lose its pathogenicity during seed transmission. Stewart *et al.* (2005) described such an evolutionary trend on *Barley stripe mosaic virus* (BSMV).

Previously, electron microscopic examination of 3-day-old rye seedlings grown from seeds collected in SBCMV infected plants revealed the presence of rod-shaped virus particles in the mesophyll cells, identified as SBCMV virions (Garbaczewska *et al.* 1997). It was concluded that the virus isolate was successfully transmitted *via* seeds in spite of previous certainty that SBCMV could not be transmitted this way. Additional data concerning the possibility of seed transmission of SBCMV was provided in subsequent years (Jeżewska 2006; Budge *et al.* 2008). However the doubts of some researchers were not definitely dispelled because only RNA2 of SBCMV was detectable in winter wheat plants grown from infected seeds. It is noteworthy that a few years earlier Clover *et al.* (1999) reported the occurrence in England of an uncharacterized virus of winter wheat, seed transmissible, which in many respects resembled SBWMV. Nevertheless its identity was not definitely determined.

Demonstration of such an effect in the case of SBWMV was limited by the sensitivity of diagnostic tools in view of a generally very low virus concentration. Results observed in the ELISA test contrasted with those obtained using IC-RT-PCR. The ELISA kit from Loewe containing monoclonal antibodies should guarantee a high specificity of the test thus producing reliable results. However it is unclear if the above demonstrated discrepancy was due to possibly false positive ELISA results or limited sensitivity of the IC-RT-PCR performed. Some disagreement between results obtained with serological and molecular diagnostic methods was already reported by Lebas *et al.* (2009). Electron microscopic examinations of crude sap preparations were previously abandoned as somewhat hazardous and thus uncertain.

In our opinion the examples of SBWMV-Pol1 detection by two methods in young wheat plants without any contact with potential external sources of the virus infection seriously suggests the possibility of seed transmission of the virus.

It is noteworthy that a list of seed transmitted viruses was systematically lengthened (Mink 1993). However, since his publication no exhaustive and upgraded review has been available. It seems that a low concentration of virus particles in plant tissues, associated for the most part with seed-borne infections, is one of several factors limiting the proper detection and documentation of how it spreads.

Acknowledgements

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