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Development of a one-step immunocapture real-time RT-PCR assay for the detection of barley stripe mosaic virus strains in barley seedlings

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Summary. – A one-step immunocapture real-time RT-PCR (IC-real-time RT-PCR) was developed for efficient detection of barley stripe mosaic virus (BSMV) in barley seedlings. The novel detection system was designed using a primer set targeting the conserved region in the triple gene block 2 (TGB2) to expand its capacity to detect all BSMV strains. This assay was evaluated for its efficiency in detecting BSMV in barley seedlings. Using the immunocapture sample preparation, real-time RT-PCR was able to detect BSMV in samples, which were indicated as negative by ELISA. The sensitivity of detection in the real-time RT-PCR was as low as 50 fg/µl of total viral RNA under optimal reaction conditions. This level of sensitivity indicated that the one-step IC-real-time RT-PCR developed in the present study could be used for routine plant and seed health assays.

Keywords: barley stripe mosaic virus; barley; IC-real-time RT-PCR; diagnostics

Barley stripe mosaic virus (BSMV) is the causal agent of a potentially serious disease in barley. BSMV, the type member of the genus *Hordeivirus*, is efficiently seed-transmitted, as well as transmitted mechanically (Hamilton and Jackson, 1995). BSMV has a tripartite genome consisting of positive strand ssRNAs designated as α, β and γ (Jackson *et al*., 1991). It has been reported to occur worldwide (Bragg and Jackson, 2004). The characteristic feature of the virus is a high level of genetic diversity, due to which different strains can cause a wide range of disease symptoms in barley (Jackson *at al*., 1991). The disease was first observed in North America in 1910 and for about 70 years remained a potential threat for barley production in the United States (Bragg and Jackson, 2004). BSMV infection with aggressive strains seriously reduces seed yield and quality (Carroll, 1980). Systematic control measures made it possible to eradicate the pathogen from barley seed stocks in the USA (Carroll, 1983). The impact of BSMV on barley production in Europe was limited, but the virus appeared on the European and Mediterranean Plant Protection Organization (EPPO) A2 quarantine list (OEPP/EPPO, 1983). It has been suggested that barley seeds should come from the seed crops, which have been verified for the presence of the virus (OEPP/EPPO, 1990), and the procedure for seed testing was recommended (OEPP/EPPO, 1991). The virus was removed from the EPPO A2 List in 1999.

In Poland, the virus was first reported in 2000 (Jeżewska, 2000). An extensive research was carried out in the years 2001–2005, aiming at the evaluation of the virus incidence in barley seeds and of the risk of yield losses in barley crops (Jeżewska, 2006). The presence of BSMV in barley seedlings was confirmed in twelve barley cultivars out of the 37 tested and in 35 breeding lines out of the 105 examined by ELISA tests. It has been observed that the viral evolution is shifting towards very mild isolates, where the level of viral proteins in the plant tissue is extremely low (latent infection) (Stewart *et al.,* 2005). Due to this fact, the serological diagnosis may not provide the sensitivity that is necessary to detect low-level virus concentrations. Detection and identification of BSMV

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Abbreviations: BSMV = barley stripe mosaic virus; BSMV-De-M = German mild isolate of BSMV; BSMV-M = Polish mild isolate of BSMV; BSMV-R = Polish aggressive isolate of BSMV; IC-real-time RT-PCR = immunocapture real-time RT-PCR; OD = optical density; OEPP/EPPO = European and Mediterranean Plant Protection Organization; TGB2 = Triple Gene Block 2

by RT-PCR would provide not only greater sensitivity, but also a quantitative assay when used with real-time technology. Moreover, RT-PCR-amplified products could be used for nucleotide sequencing to establish genetic relationships with other strains.

In this study, three different methods were evaluated in terms of their capacity to detect BSMV in barley seedlings. In addition, the method of IC-real-time quantitative RT-PCR was developed for BSMV detection and immunocapture sample preparation was incorporated to complete the entire test, from sample preparation to amplicon detection, in a single tube.

Polish mild BSMV-M and aggressive BSMV-R isolates (Jeżewska, 2006) were used in this study. The isolate designated as BSMV-De-M was kindly provided by Dr. Frank Rabenstein from Julius Kühn Institut (BAZ) in Quedlinburg (Germany). BSMV-R and BSMV-De-M were used as reference materials. The isolates were propagated in barley cv. Annabell by mechanical inoculation and maintained under standard glasshouse conditions. Seeds collected from BSMV-M-infected barley plants were sown in a greenhouse and used for further tests. Three-week-old seedlings originating from these seeds were tested by the Indirect ELISA method (Clark and Adams, 1977). The test was performed using 1000x diluted anti-BSMV γ-globulin (1 mg/ml) (produced in Research Institute of Pomology, Skierniewice, Poland) and 10000x diluted universal anti-rabbit conjugate (Sigma-Aldrich). Absorbance at 405 nm was measured with an ELISA reader (ELx 800, BioTek Instruments). A sample was considered positive when its optical density (OD) value was at least two-times higher than the average OD of the negative control (healthy plants). Two hundred and fifty collected seeds were divided into sets of fifty samples. BSMV-R and BSMV-De-M were used as positive controls. Absorbance value obtained from negative and positive controls amounted to 0.15 and 1.4, respectively. Seven samples with an OD value above 0.2 suggested the possibility of virus infections, whereas the others were clearly negative.

The RT-PCR and IC-real-time RT-PCR methods were developed in order to verify the presence of BSMV in barley seedlings. The methods were optimized using total RNA from BSMV-R, BSMV-De-M and BSMV-M. Total RNA was extracted from 100 mg of infected plant leaves using the NucleoSpin® RNA Plant (Machery-Nagel) according to the manufacturer's instructions. The RNA was measured using a NanoDrop spectrophotometer (ThermoScientific) and adjusted to 50 ng/µl. Isolated RNA was subsequently used in RT-PCR with primers designed by Estabrook (1998) and Torrance (2006). A new primer set TGB2F (5'-GGATGAAGACCACAGTTGGTTC-3') and TGB2R (5'-CTAGCCAATATCGCATAGTAATG-3') was designed using the Oligo 6.0 software based on the alignment of the TGB2 gene of different BSMV sequences retrieved from the GenBank Database. The primers correspond to the nucleotides 2309–2706 of RNAβ of the BSMV-Norwich isolate (Acc. No. JF803285). First-strand cDNA of BSMV was synthesized using SuperScript ® III First-Strand Synthesis SuperMix (Invitrogen) with the reverse primer REV (5'-AGCTTCGGCTCAGTATGCACAC-3'), complementary to the nucleotides 3191–3212 of RNAβ of the same isolate. Subsequently, PCR was carried out in a 20 µl reaction volume, using 2 µl cDNA and the AccuPrime™ Taq DNA Polymerase System (Invitrogen) reaction mix with a TGB2F/R primer pair. 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 the final cycle of 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 in UV. The PCR products were eluted using NucleoSpin® Gel and PCR Clean-up (Machery-Nagel), ligated into the pGEM T-Easy system cloning vector (Promega), and *Escherichia coli* TOP10 competent chemical cells (Invitrogen) were transformed with the obtained constructs. Plasmid DNA was isolated using NucleoSpin® Plasmid (NoLid) (Machery-Nagel). At least two independent cDNA clones were sequenced with universal primers M13F and M13R. The obtained nucleotide sequences were analyzed using the BlastN program, compiled and edited in the BioEdit software (Hall, 1999), and deposited in the GenBank Database under Acc. Nos.: KC967290-91 and KF010869. No RT-PCR products were obtained with primers designated based on literature. This might suggest the presence of changes in the nucleotide sequence of the Polish BSMV isolates in the region of primers hybridization.

De novo designated primers TGB2F/R generated an expected product of 397 bp encompassing the coding region of TGB2 in all samples tested. A comparison of nucleotide sequences of the Polish BSMV isolates revealed 98%–99% nucleotide sequence identity with other isolates described to date. It confirmed that the TGB2 region is rather conservative and primers used may be applied for the detection of a wide range of BSMV isolates. Subsequently, designed primers were tested by real-time RT-PCR. The reaction was run in a mix containing 0.4 µmol/l of each TGB2F and TGB2R primers, 12.5 µl of the Brilliant II SYBR® Green QRT-PCR 1-Step master mix (Agilent Technologies), 1 µl of the RT/RNase Block enzyme mix and 50 ng of total RNA in a total volume of 25 µl. The thermal conditions were as follows: 1 cycle of 50°C for 30 min, followed by 1 cycle of 95°C for 10 min, then 25 cycles of 95°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec, and next 1 cycle of 95°C for 1 min, 1 cycle of 76°C for 30 sec and 1 cycle of 95°C for 30 sec. The RNA isolated from healthy plants served as a negative control. The thermal cycling process and fluorescence signal detection were carried out with the Mx 3005P Stratagene System. The sensitivity of real-time RT-PCR and RT-PCR was

evaluated using a range of 10-fold dilutions (from 50 ng/µl to 50 fg/µl) of total RNA of BSMV-R. As shown in Fig. 1, the PCR product was detected in RT-PCR with the concentration of the template as low as 5 pg/µl, whereas real-time RT-PCR was capable of detecting viral RNA at as little as 50 fg/µl of total RNA. With this knowledge, we decided to focus on real-time RT-PCR, as it turned out to be more sensitive, and combine it with immunocapture, as it does not require RNA isolation. This makes the whole procedure less time- and money-consuming.

IC-real-time RT-PCR was conducted using the samples tested previously by ELISA. Briefly, PCR tubes were coated with 25 µl of anti-BSMV γ-globulin diluted 25-times in the coating buffer (pH 9.6) and incubated at 37°C for 1 hr. The plant sap was loaded in the anti-BSMV antibody-coated tubes and incubated at 37°C to allow BSMV particles to

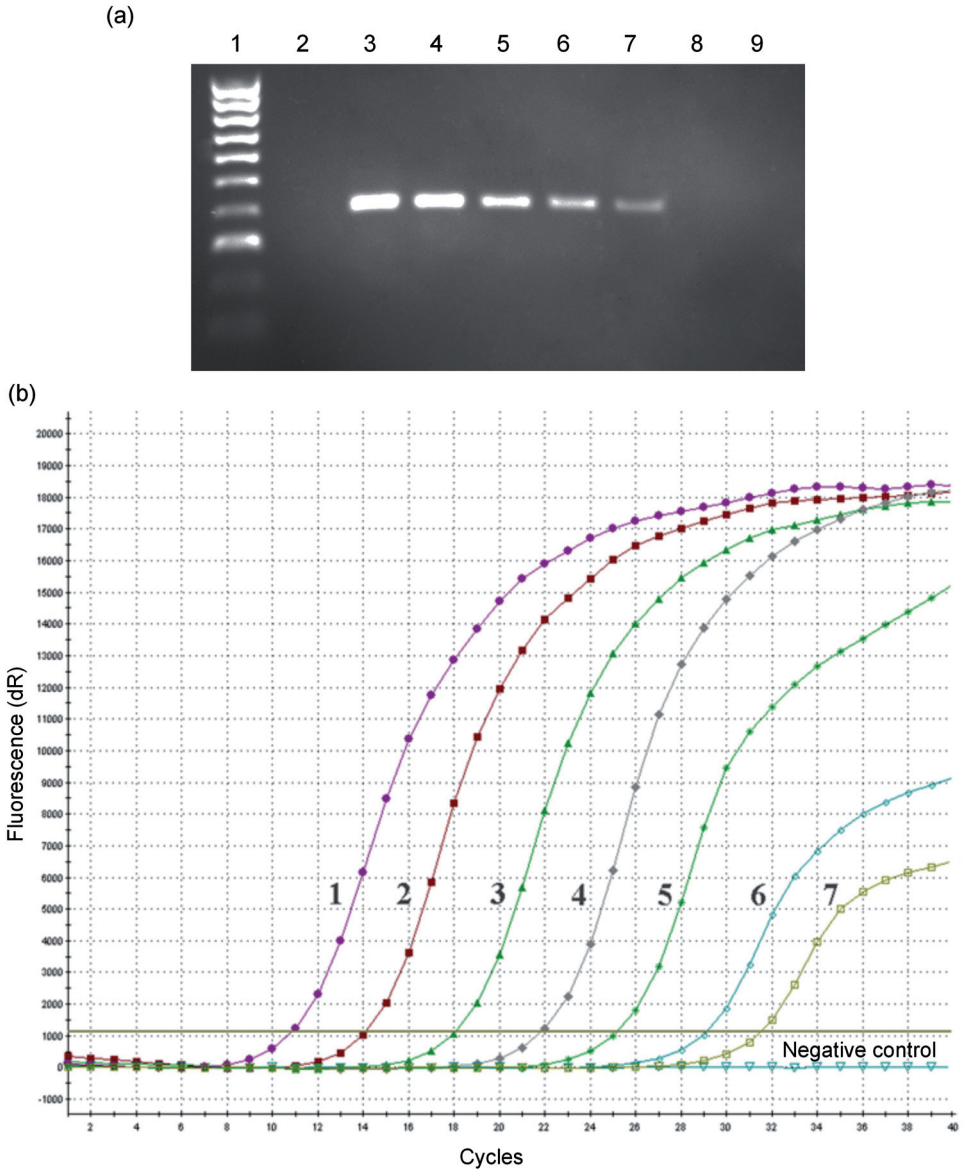

Fig. 1

The detection limit of BSMV in RT-PCR and real-time RT-PCR assays using a series of dilutions of total RNA (starting at 50 ng/µl)
a) Electrophoretic separation of RT-PCR products in 1% agarose gel. Line 1 – DNA Ladder Nova 100bp (Novazym), line 2 – negative control, lines 3–9 ten-fold dilutions of total RNA. b) Sensitivity of real-time RT-PCR assay as monitored by amplification curves. Amplification plots (1–7) correspond with ten-fold dilutions of total RNA.

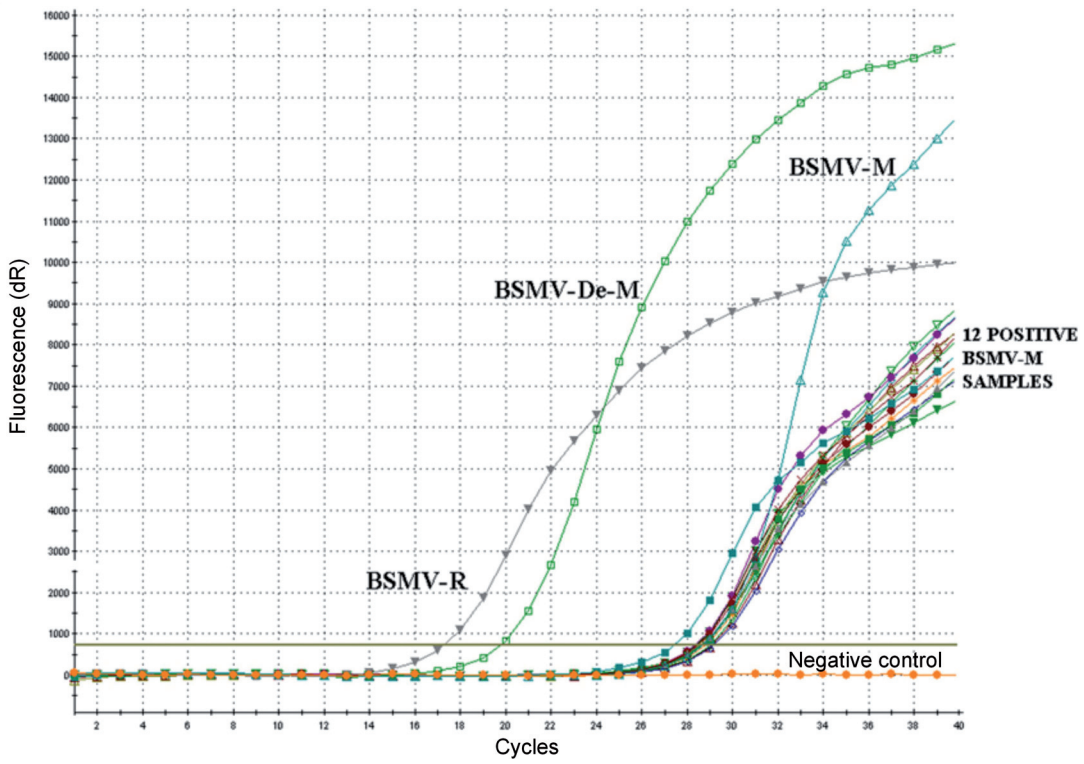

Fig. 2

The detection of BSMV in barley seedling by the IC-real-time RT-PCR assay
Amplification plots correspond with BSMV tested samples.

be trapped onto the tubes. After washing with phosphate buffer (pH 7.4), treated PCR tubes were ready for real-time RT-PCR. The reaction was run under the same thermal conditions as described above. Out of the 50 samples tested, 12 were positive for the presence of BSMV (Fig. 2). The C_t value obtained for the aggressive isolate was about 20 and for seedling samples $C_t = 29$, which indicated a lower virus titer in the sap. The analysis confirmed seven uncertain samples from the ELISA assay to be positive. Moreover, five more samples were detected as positive. It confirmed that the IC-real-time RT-PCR is a more sensitive method, able to detect even a very low virion concentration, undetectable with other standard techniques. To verify the specificity of the IC-real-time RT-PCR, the obtained positive samples were purified and sequenced as well. The sequencing revealed a 99–100% sequence identity with other BSMV sequences deposited in the GenBank.

Pathogen diagnosis plays an important role in crop improvement and disease management. So far, mainly serological tests like ELISA (Faris-Mukhayyish and Makkouk 1983; Torrance 1998), immunogold technique (Hsu, 1984), filter paper sero-assay (FiPSA) (Haber and Knapen, 1989) and only few molecular techniques for BSMV detection (Estabrook *at al.*, 1998; Torrance *at al.*, 2006; Ay *et al.*, 2008) have been published. All of these publications refer to aggressive isolates of BSMV. The aim of this study was to evaluate the applicability of molecular biology techniques to the detection of BSMV in seedlings. The ELISA method was shown to be an inadequate tool in diagnostics of mild BSMV isolates. The TGB2F/R primers used in this study were useful for the detection of all BSMV isolates in both conventional and real-time RT-PCR. The designated primers could be widely applied for the detection of different BSMV isolates, as they amplified a conserved region of the viral genome. It is worth mentioning here that primers available in the literature were not capable of detecting the Polish BSMV isolates. A comparison of conventional RT-PCR and real-time RT-PCR revealed that the real-time RT-PCR detection limit was 100 times higher than in RT-PCR. To avoid the necessity of RNA isolation we developed a new technique based on real-time PCR for the detection of BSMV. IC-real-time RT-PCR is a combination of immunology and RT-PCR, where the specific antibody captures the viral antigen (in the form of virions), followed by the RT-PCR reaction. The initiation of the reaction by heating leads to the dissociation of virions and to the release of the genomic RNA.

Thus, the specificity and sensitivity of this method is very high. In the present study we clearly demonstrated that the IC-real-time RT-PCR developed was capable of detecting BSMV in the samples, which were indicated as negative by the ELISA test.

In conclusion, the IC-real-time RT-PCR is the most reliable and sensitive test for BSMV detection and it is easier and quicker than standard ELISA or RT-PCR tests. This IC-real-time RT-PCR performed using TGB2F/R primers could be a useful tool for BSMV screening tests, where high sensitivity, reliability, speed and quantitative data are required.

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