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RAPID COMMUNICATION

Development of reverse transcription-loop-mediated isothermal amplification assay for the detection of genetically different isolates of maize dwarf mosaic virus

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Abstract

Maize dwarf mosaic virus (MDMV) is a serious and widespread virus pathogen of maize plants. This +ssRNA virus belongs to the *Potyvirus* genus in the Potyviridae family. Together with sugarcane mosaic virus (SCMV) it causes one of the most important viral diseases on maize crops in the world – maize dwarf mosaic. Both viruses are transmitted in the same non-persistent manner by several aphid species. They induce similar symptoms of leaf mosaic or mottling, stunting and a reduction in plant weight and grain yield. Available MDMV diagnostics include primarily commercialized enzyme-linked immunosorbent assays (ELISA) and reverse transcription-polymerase chain reactions (RT-PCR). Here, labor-saving reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay was optimized for identification of genetically different MDMV isolates. For this purpose, primer sets, MDMVF3/MDMVB3 and MDMVFIP/MDMVBIP amplifying fragments of coat protein coding sequence of MDMV, were used. The specificity of the reaction was verified using three MDMV (-P1, -Sp, -PV0802-DSMZ) and three SCMV (-P1, -PV0368--DSMZ, -PV1207-DSMZ) isolates. Obtained products were visualised by DNA staining, electrophoretic separation as well as by real-time monitoring of the reaction. The sensitivity of RT-LAMP and conventional RT-PCR reactions was comparable. Both methods could detect virus as low as 550 fg · μl^{-1} of total RNA. This technique has application value for screening MDMV by phytosanitary services.

Keywords: MDMV, SCMV, identification, maize, RT-LAMP technique

Maize dwarf mosaic virus (MDMV) belongs to the *Potyvirus* genus, in the Potyviridae family (Adams *et al.* 2012). MDMV together with sugarcane mosaic virus (SCMV) causes one of the most important viral diseases on maize crops in the world – maize dwarf mosaic. MDMV is transmitted in a non-persistent manner by several aphid species and at a low rate (0.4%) by seeds (Ford *et al.* 1989). It infects over 200 grass species, including Johnsongrass (*Sorghum halepense* L. Pers) which is its overwintering host and virus reservoir (Achon *et al.* 2011). MDMV induces leaf mosaic or mottling which may appear only on the upper leaves. In the case of early infection of susceptible maize varieties, plant height reduction, delayed maturity and increased butt blanking lead to yield losses up to 70% (Gordon 2004). The virus was first reported in 1960 in Ohio and then in more than 37 states in the USA (Wijayasekara and Ali 2021). In general, the virus presence was confirmed in North, Central and South America and Europe (Gordon 2004). In Poland MDMV was first detected in 2005 (Trzmiel and Jeżewska 2006) and in the following years in several regions of the country (Trzmiel 2008; Trzmiel and Jeżewska 2008).

MDMV has monopartite +ssRNA genome of ~9.5 kb in length with a covalently linked 5'-terminal protein (VPg) and a 3'poly(A) tail on 3' end (Kong and Steinbiss 1998). A single open reading frame encodes polyprotein which is proteolytically

cleaved by self-encoded proteinases on the final 10 proteins: the first proteinase (P1), helper component proteinase (Hc-Pro), the third protein (P3), 6 kDa protein 1 (6 K1), cytoplasmic inclusion protein (CI), 6 kDa protein 2 (6 K2), nuclear inclusion protein "a" including VPg (NIa-VPg), NIa proteinase (NIa--Pro), nuclear inclusion protein "b" (NIb), coat protein (CP) and PIPO. The recently published results of evolutionary relationships among MDMV isolates, based on their complete genome sequences, showed their grouping into G1 and G2 phylogroups. The European isolates belong to the G2 group (Wijayasekara and Ali 2021).

To date, available MDMV diagnostics have been based on tissue print-immunoblotting (TPIB) (Hohmann *et al.* 1996), commercialized indirect- or double-antibody sandwich enzyme-linked immunosorbent assays (Indirect-ELISA, DAS-ELISA), competitive radioimmunoassay (RIA), dot blot immunoassay, sodium dodecyl sulfate (SDS) immunodiffusion test, reverse transcription – polymerase chain reaction (RT-PCR), combined RT-PCR with electrochemiluminescence and DNA microarray (Maizepath)-based detection (Kannan *et al.* 2018). All of the above-mentioned techniques can be successfully replaced by reverse transcription-loop-mediated isothermal amplification of nucleic acid (RT-LAMP) reaction which has been developed as a fast and effective diagnostic method for many RNA viruses (Panno *et al.* 2020). In RT-LAMP assay nucleic acids can be specifically amplified under isothermal conditions (63–65°C) in a relatively short time. RT-LAMP is a cost-effective alternative to RT-PCR, as the reaction can be carried out in a heat block or a water bath. It has been shown that RT-LAMP exhibits similar or even higher sensitivity than the conventional RT-PCR (Hasiów-Jaroszewska and Borodynko 2013; Anandakumar *et al.* 2020; Li *et al.* 2022). Taking into account the advantages of the isothermal assay, the main aim of this study was to develop and optimize RT-LAMP with originally designed primers, for rapid detection of genetic variants of MDMV isolates. To our knowledge, this is the first diagnostic assay based on RT-LAMP for MDMV.

In this study three MDMV and three SCMV isolates were used. MDMV-P1 (EU240460) and SCMV--P1(EU761241) originated from Poland while MDMV--Sp (AM110758) from Spain, (kindly provided by Dr Maria Angeles Achon from the University of Lleida (UdL)). Additionally, three available commercial MDMV-PV-0802-DSMZ, SCMV-PV-0368-DSMZ and SCMV-PV-1207-DSMZ isolates were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Brunswick, Germany). The virus isolates were propagated by mechanical inoculation of young plants (12–13 in BBCH scale) of sweet maize cv. Waza and fodder maize cv. Polonez with 0.05 M phosphate buffer pH 7.0. The plants were maintained in closed mesh cages, under standard greenhouse conditions (light/dark cycle 16 h/8 h at 23°C). Total RNA was isolated using a RNeasy Mini Kit (Qiagen, Hilden, Germany), approximately 21 days after inoculation, following the producer's procedure. The quality and concentration of extracted RNAs were assessed using a NanoDrop 2000 spectrophotometer (Nonodrop Technologies, Delaware, USA). In the first step of this study, complete nucleotide sequences of the CP region of commercial MDMV and SCMV isolates were amplified with two newly designed primer pairs: MDMVcp-F/MDMVcp-R (5'GTCTATGCACGACAATT CTTCG3'/5'CTAAATTACTGCGTAAAAG3') and SCMVcp-F/SCMVcp-R (5'GGATACGTAGAAG ACTACAATG3'/5'ATTATAGGTACTGCAAACAG3') and Transcriptor OneStep Kit (Roche, Basel, Switzerland). The oligonucleotides were designed with Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) (Rosen and Skaletski 2000) based on the nucleotide sequence of Bulgarian MDMV (AJ00169) and Spanish SCMV (AM110759) isolates. RT-PCR reactions were carried out in a total volume of 25 µl with 1× reaction buffer, 0.4 µM primers, 0.5 µl of transcriptor enzyme mix, and 1 µl of template RNA. The thermal conditions of the reactions were as follows: reverse transcription at 50°C for 30 min, followed by initial denaturation at 94°C for 7 min then 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 68°C for 60 s and a final elongation at 68°C for 7 min. The specific RT-PCR products, of the appropriate size of 921 and 1013 bp, respectively, were excised from agarose gels, purified with Wizard® SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI, USA) and then used for direct sequencing with the above-mentioned specific primers by Genomed S.A. (Warsaw, Poland). The obtained nucleotide sequences were analyzed using Standard Nucleotide BLAST (BLAST, http://blast.cnbi.nlm.nih.gov/Blast.cgi), compiled and edited using BioEdit software (Hall 1999). Complete CP sequences of MDMV-PV--0802-DSMZ (OM807129), SCMV-PV-0368-DSMZ (OM857559), SCMV-PV-1207-DSMZ (OM857560) isolates were deposited in the NCBI GenBank database. In order to identify conserved and variable regions of MDMV and SCMV *CP* gene multiple sequence alignments were performed using the Polish and the Spanish isolates and others retrieved from GenBank (e.g., FM883164, JQ403608-09, MW026053-68, MH093733--34, AJ310102-05, JX047382-99) in ClustalW (Thompson *et al.* 1994). Based on obtained results, diagnostic primers group (Table 1) were designed using LAMP Designer software (OptiGene, Horsham, UK). The specificity and selectivity of RT-LAMP primers was verified using total RNA samples extracted from maize plants infected with the above-mentioned MDMV and SCMV isolates. RT-LAMP assay was carried out in

Table 1. Primers used in RT-LAMP

Primer name	Type	Genome position*	Primer sequence (5′- 3′)
MDMV F3	forward outer	9074-9091	TCATCTCGAACTCCAGCA
MDMV B3	reverse outer	9329-9311	ACAACCTCACCACAATAGC
MDMV FIP (F1c+F_2)	forward inner primer	9211-9191/ 9144-9161	AGCTGTGTGGCGTTCTGTAT-TACACACGGATGTTTGGTC
MDMV BIP (B1c+B_2)	backward inner primer	9215-9236/ 9279-9260	GACGTTAGTCGCAACATCCACT-AGTTGAACCCCGTATCAATG

*genome position according to the reference sequence of MDMV (Acc. no. AJ001691)

a total volume of 25 µl. The reaction mixture consisted of 15 µl of Isothermal Mastermix ISO-001nd (Novazym, Poznan, Poland), 4 µl of 10 µM MDMV-FIP and MDMV-BIP mix, 1 µl of 10 µM MDMV-F3 and MDMV-B3 mix primers (Table 1), 0.25 µl of LAMP reverse transcriptase (1 U/µl) (Novazym), 1 µl of template RNA and 3.75 µl of sterilized water. RNA sample extracted from healthy maize plant and water sample added to the reaction mix were used as the negative controls. The optimal conditions of RT-LAMP assay were determined using various ranges of temperature and time. The tubes were incubated at 60–63°C for 30–60 min in a thermoblock (Biometra, Göttingen, Germany). The RT-LAMP products were checked on 1.5% agarose gel staining with Midori Green DNA Dye (NIPPON Genetics Europe GmbH, Düren, Germany) as well as by direct evaluation of color change under UV light after the addition of 2 µl of EvaGreen Dye (Biotium, Hayward, CA, USA). Additionally, the same RT-LAMP test was performed using Isothermal Mastermix ISO-001 (Novazym) in a LightCycler 96 Instrument (Roche) to analyze amplification curves under real-time conditions. The fluorescence data was quantified on the FAM channel (excitation at 470 nm, detection at 510 nm) for 40 min. The final step of this study was to evaluate and compare the sensitivity of RT-LAMP assay and conventional RT-PCR. For this purpose, 1 µl of 10-fold serial dilutions (from 10^–1 to 10^–6) of total RNA of maize plants infected with MDMV-PV0802-DSMZ (initial concentration ~550 $\mu g \cdot \mu l^{-1}$) were used as template for the reactions, as described above. RT-PCR was carried out with Transcriptor OneStep Kit (Roche) and Mreal-F/Mreal--R primers (5'GAGTCCACGGGAAAAGACAA3'/5'TATCGAATTCAGCCCTGGTT3'), designed previously by Trzmiel, under thermal conditions described by the author (Trzmiel 2009). The limit of detection (LOD) of both compared techniques was estimated from the results of 1.5% agarose gel electrophoresis.

Effective and rapid diagnostics are important key steps in effective prevention and control of viral diseases. MDMV and SCMV induce similar symptoms and cause the same viral disease, therefore, specific diagnostic tools are required to distinguish between both viruses. To date, RT-LAMP assay has been successfully optimized for the detection of different viruses infecting ornamental plants (Yao *et al.* 2022), vegetables (Hasiów-Jaroszewska and Borodynko 2013; Budziszewska *et al.* 2016), cereals (Zarzyńska-Nowak *et al.* 2018), sugarcane (Keizerweerd *et al.* 2015; Anandakumar *et al.* 2020) and maize (Chen *et al.* 2017; Li *et al.* 2022). Due to genetic differences between MDMV and SCMV, the primer pairs for the RT-LAMP assay were originally designed based on the alignment of *CP* gene sequences. The RT-LAMP test optimized in this study was capable of detecting only genetically different MDMV-P1, -SP, -PV-0802-DSMZ and not SCMV-P1, -PV-0368-DSMZ, -PV-1207-DSMZ isolates. The presented results indicated 63°C as the optimal reaction temperature. Moreover, no difference was observed between tested reaction times (data not shown), thus 63°C and 40 min were chosen as the optimal conditions of RT-LAMP. The amplified products, visible on agarose gel as ladder-like DNA fragments, were obtained only for tested MDMV samples whereas no amplicons were received for SCMV samples, total RNA probe isolated from healthy maize plant as well as for no template control (NTC) (Fig. 1A). Moreover, the green color visible in UV light was observed after adding the dye only to MDMV samples and no color changes were visible for the negative controls (SCMV, healthy plant and NTC) (Fig. 1B). Similarly, analysis of real-time RT-LAMP showed amplification curves only for MDMV samples. The amplification plots were observed 15 to 20 min after the onset of the reaction (Fig. 1C). The results obtained in this study confirmed that optimized RT-LAMP reaction is highly specific and can be used to distinguish between MDMV and SCMV infections. Contrary to the diagnostic methods available so far (Kannan *et al.* 2018) RT-LAMP results were available in less than 1 h. Moreover, the results of this study revealed that the sensitivity of the presented technique was comparable with conventional RT-PCR reaction. Both RT-LAMP amplicons and RT-PCR product (199 bp in size) were detectable up to 550 $fg \cdot \mu l^{-1}$ ($55 \mu g \cdot \mu l^{-1} \times 10^{-6}$) of total RNA (Figs 2A

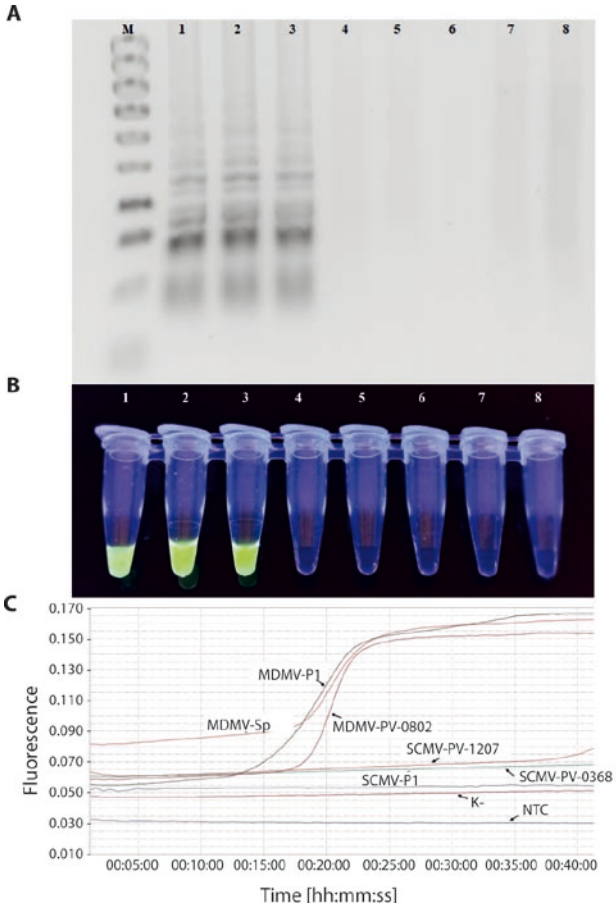

Fig. 1. Detection of MDMV isolates by RT-LAMP: (A) – analysis of RT-LAMP products on agarose gel; Lane M –100-bp DNA ladder (Novazym, Poznań, Poland); Lane 1 – MDMV-P1; Lane 2 – MDMV--Sp; Lane 3 – MDMV-PV0802; Lane 4 – SCMV-P1; Lane 5 – SCMV--PV0368; Lane 6 – SCMV-PV1207; Lane 7 – negative control (sap of healthy maize); Lane 8 – NTC (water); (B) – direct detection of RT-LAMP products using EvaGreen stain (Biotium). The numbering from panel A has been preserved; (C) – evaluation of RT-LAMP results in real-time conditions

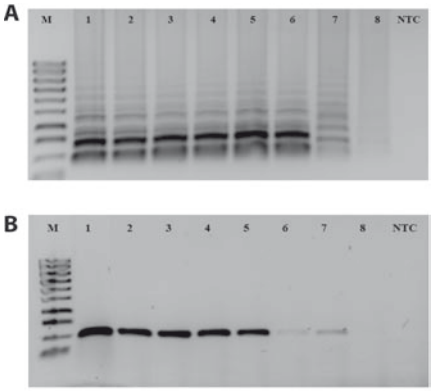

Fig. 2. A summary of the results on the sensitivity of MDMV detection by RT-LAMP (A) and RT-PCR tests (B) using 10-fold serial dilution of template RNA. Concentrations of RNA were as follows: Lane 1 – 550 μg · μl^{-1}; Lane 2 – 55 μg · μl^{-1}; Lane 3 – 5.5 μg · μl^{-1}; Lane 4 – 550 ng · μl^{-1}; Lane 5 – 55 ng · μl^{-1}; Lane 6 – 5.5 ng · μl^{-1}; Lane 7 – 550 fg · μl^{-1}; Lane 8 – 55 fg ng · μl^{-1}; Lane M –100-bp DNA ladder (Novazym); Lane NTC – negative control (water)

and B). The sensitivity of the described RT-LAMP assay corresponded with that presented for maize rough dwarf disease. Du *et al.* (2019) found the same sensitivity level of RT-LAMP, dilution up to 10^{-6} μg per reaction. In conclusion, our newly developed technique offers significant time and cost-saving advantages. Furthermore, according to our knowledge it is the first report presenting the use of the RT-LAMP assay for MDMV detection. This useful diagnostic tool can be implemented for rapid identification of MDMV infection in maize crops.

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