DEVELOPMENT OF A REAL TIME RT-PCR ASSAY FOR DETECTING GENETICALLY DIFFERENT *PEPINO MOSAIC VIRUS* ISOLATES

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Abstract: Over a period of few years, *Pepino mosaic virus* (PepMV) has become one of the most important viral pathogen in tomato production worldwide. So far, five PepMV genotypes (EU, LP, CH2, US2 and US1) have been detected. A real time reverse transcription polymerase chain reaction (RT-PCR) procedure, using the fluorescence dye SYBR Green was developed for a rapid and reliable detection of genetically diverse *Pepino mosaic virus* isolates. This procedure was used for the detection and identification of PepMV in both *Solanum lycopersicum* and *Nicotiana benthamiana* species. The melting temperature (Tm) for members of a particular strain was very similar, with a host effect that did not hinder strain identification. Under optimal reaction conditions, sensitivity of the detection was as low as 100 fg of viral RNA from infected plants. This level of sensitivity indicated that the real time RT-PCR developed in the present study could be used for routine plant health assays.

Key words: real time RT-PCR, PepMV, genetic diversity, detection

INTRODUCTION

   Pepino mosaic virus, a member of *Potexvirus* genus was found for the first time in 1974 in Peru on pepino (*Solanum muricatum*). The presence of PepMV in Europe was confirmed in 1999 and the virus proved to be a very dangerous pathogen for tomato. The virus was also detected in tomato crops in North and South America. The isolates of PepMV represent five genotypes (EU, LP, CH2, US2 and US1) (Maroon-Lango *et al.* 2005; Ling 2007; Hanssen *et al.* 2008; Hasiów *et al.* 2008). Genome sequence analyses of numbers of PepMV isolates indicated significant polymorphism within the species.

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In Poland, we have collected from different regions of the country several isolates of PepMV which differ in phenotypic and genetic properties. Phylogenetic analysis, based on two parts of the genomes: triple gene block (TGB) and coat protein gene (CP), revealed that Polish isolates belonged to two genotypes: EU (for example PepMV-SW) and Ch2 (for example PepMV-PK and PepMV-Pa) (Pospieszny *et al.* 2007; Pospieszny *et al.* 2008; Hasiów *et al.* 2008). The isolates induced different symptoms on tomato plants, from mild mosaic (PepMV-PK) to severe necrosis and plant death (PepMV-Pa). The virus spreads very fast by mechanical transmission. Diagnosis of PepMV based on disease symptoms is not reliable because not all PepMV infected plants show symptoms. Until now, a set of primers which amplified different regions of PepMV genomes have been designed for standard reverse transcription polymerase chain reaction (RT-PCR) (Pagen *et al.* 2006). Using the primer set Pagan *et al.* (2006) we were not always able to obtain positive results for all genotypes identified to data. In addition, there is a need for rapid and sensitive method for the detection of PepMV in tomato seeds as PepMV is believe to be a seed-borne pathogen.

In this report we present a real time RT-PCR assay which enables the discrimination and detection of genetically diverse PepMV isolates.

MATERIALS AND METHODS

Virus sources and plant material

Nicotiana benthamiana and tomato (*Solanum lycoperiscum*) plants were grown in a growth chamber (21°C, 16 h photoperiod) prior to inoculation. The primary sources of inoculum were PepMV-infected tomato plants collected in Poland (two genotypes; PepMV-SW as European and PepMV-PK/Pa as Ch2). Tomato and tobacco plants were mechanically inoculated with each isolate for propagation. Young tomato leaves showing severe symptoms were ground in 0.05 M phosphate buffer pH 7.0 and the extract was mechanically inoculated onto carborundum dusted leaves of the test plants. Inoculated plants were maintained in the greenhouse and monitored for symptom development. The same volume of inoculum was used on each plant. Young leaf tissue was then selected for real time assays because it is a good source of RNA. The presence of PepMV in the original host or in experimental test plants was checked by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using commercial polyclonal antiserum (DSMZ, Braunschweig, Germany) according to manufacturer's instructions.

Total plant RNA sample preparation

Total plant RNA was extracted from a sample of 50–100 mg leaf tissue using the RNAeasy Kit (Qiagen). The concentration was determined both in spectrophotometer and using agarose gel electrophoresis.

Primers' design

Due to the existence of genetic diversity in PepMV population it is even more critical to select an appropriate genome sequence region to design primers that would produce reliable results of RT-PCR for all virus isolates. The most important requirement was to select sequences that were conserved among all identified isolates. Analysis of multiple sequence alignment using Clustal W of the 13 available PepMV isolates

showed a region in coat protein gene suitable for primers' design. The primers F5 5'G ACTTCTCAAATCCTAATACAGC 3' and R5R 5'CACATCAGCATAAGCACGAGC 3' were designed using Oligo Analyzer (http://www.uku.fi/~kuulasma/OligoSoftware) for amplification of 166 bp fragment of coat protein gene.

Quantification

In order to evaluate the sensitivity of real time RT-PCR system for PepMV detection, a series of 10-fold dilutions of total plant RNA was prepared ranging from 100 ng/µl to 100 fg/µl. To generate a standard curve, three replicates of each concentration were amplified. In each plate, a dilution series of the RNA standard for the respective virus was run along with the negative samples for a corresponding virus. Each sample had 2–3 replicates and all reactions were repeated 3 times independently to ensure the reproducibility of the results.

The amplification cycle at which the emission intensity of the amplification product rises above an arbitrary threshold level (C_T cycle) is inversely proportional to the logarithm of the initial number of target sequences (Higuchi *et al.* 1993).

Standard curves based on threshold value (C_T) for dilution series of the RNAs were constructed. Quantification of PepMV in the plant samples was performed comparing C_T values of each sample to the C_T values of the standard regression line.

RT-PCR

Total RNA was isolated from leaves material and purified with phenol-chloroform. RNA was dissolved in 40 µl of sterile water and 1 µl of solution was used for cDNA synthesis. Reverse transcription (RT) was performed in the presence of oligodT$_{22}$ primer and M-MuLV RT (Fermentas) according to manufacturer's instructions. cDNA fragments compassing a part of CP gene was further amplified by polymerase chain reaction (PCR), using Taq DNA polymerase (Fermentas) and F5 and R5R primers. The PCR mixtures contained 2 µl template cDNA, 1 µl (in 10 µM stock) of each amplification primer, 10 mM each dNTP, 1U *Taq* DNA polymerase (Fermentas) 1.5 mM MgCl$_2$, 50 mM KCl, 10 mM Tris-HCl pH 8.3 in a total volume of 50 µl. The amplification was for 30 cycles each consisting of 30 s at 94°C, 30 s at 47°C and 20 s at 72°C and followed by a final extension step for 5 min at 72°C. RT-PCR products were examined by electrophoresis in 1% agarose gel.

Real Time RT-PCR

Real time RT-PCR was evaluated with total plant RNA as described above using QRT-PCR Master Mix SYBR Green (Stratagene) following vendor's instructions. For each 25 µl reaction, 1 µl of each primer (in 10 µM stock) and 1 µl of a template were added to 12,5 µl of Master Mix, 1 µl of enzyme mix. The thermal cycling process and fluorescence signal detection were carried out with the Mx3005P real-time PCR system (Stratagene). The cycling parameters were: reverse transcription for 30 min at 50°C, denaturation for 10 min at 95, followed by 35 cycles of 10 s denaturation at 95°C, 30 s for annealing at 64°C and 30 s at 72°C for extension. The melting curves were generated at 70°C by 30 s. As negative samples we used both healthy plants and samples without RNA.

RESULTS AND DISSCUSION

Real time RT-PCR is an accurate and sensitive method, able to detect a wide range of field isolates. It also eliminates post-PCR processing of PCR products (which is necessary in conventional PCR). This helps to increase the throughput, reduces the chance of carryover contamination and disables post-PCR processing as a potential source of error. The real-time PCR system is based on the detection and quantitation of a fluorescent dye.

The analytical sensitivity of SYBR Green RT-PCR was determined by using a serial dilution of PepMV-SW and PepMV-PK RNAs as a template for amplification. The number of RNA copy was calculated using the concentration value and Avogadro's constant. The serial dilution of the RNAs showed the expected single amplicons. The predicted RT-PCR product (length 166 bp) was confirmed by both the melting curve and agarose gel electrophoresis (Fig. 1). Non-specific products or primer-dimers were observed in our experiments (Fig. 2a, 2b). A linear relationships between the input RNA and the C_T values with regression coefficient (r2) about 0,99 were obtained for both genotypes (Fig. 3). We were able to detect about 100 fg of viral RNA for both genotypes both in *S. lycopersicum* and *N. benthamiana* which is equal to $2,71x10^3$ copies of RNA.

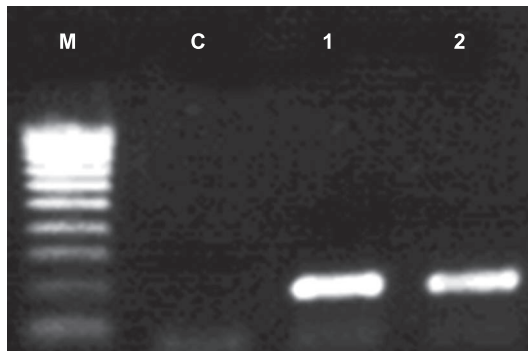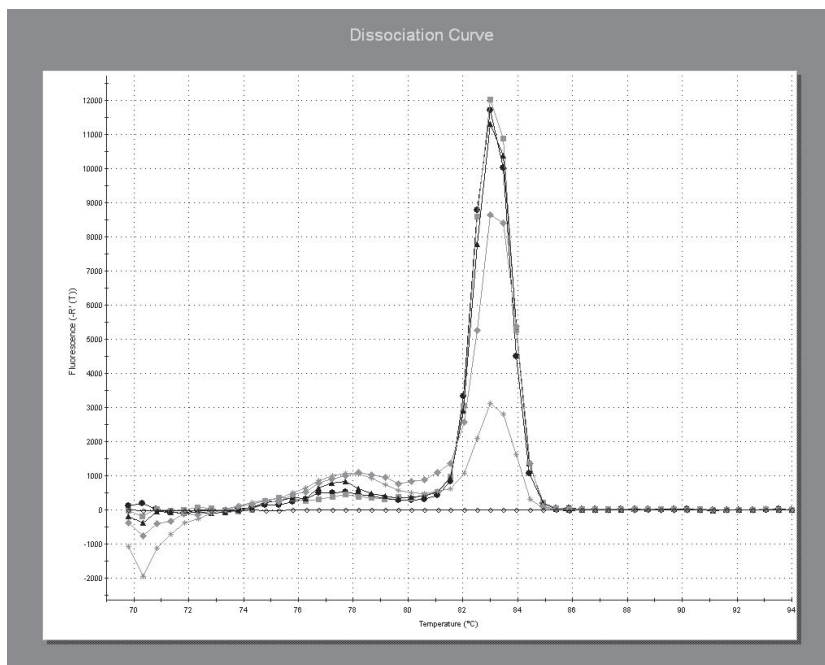
Fig. 1. Electrophoresis mobility of RT-PCR products
        M-marker HyperLadder IV (Bioline)
        C-negative control (sample without cDNA)
        1-PepMV-PK
        2-PepMV-SW

A real time RT-PCR assay for diagnosis of PepMV infection has many advantages over conventional methods such as biological indexing on *S. lycopersicum* plants, ELISA or conventional RT-PCR. The virus infectivity test is time consuming and may take even 3–4 weeks to complete. However, for a tomato seed sample within an extremely low viral contamination ELISA may not be sensitive enough. Although a sensitive, molecular method for PepMV detection based on conventional RT-PCR has been developed (Pagan *et al.* 2006) the primers developed in those studies are no longer able to reliably detect recently emerging genetically diverse isolates such as US1, Ch2 and Ch1 types. In addition, the occurrence of recombination between the EU and Ch2
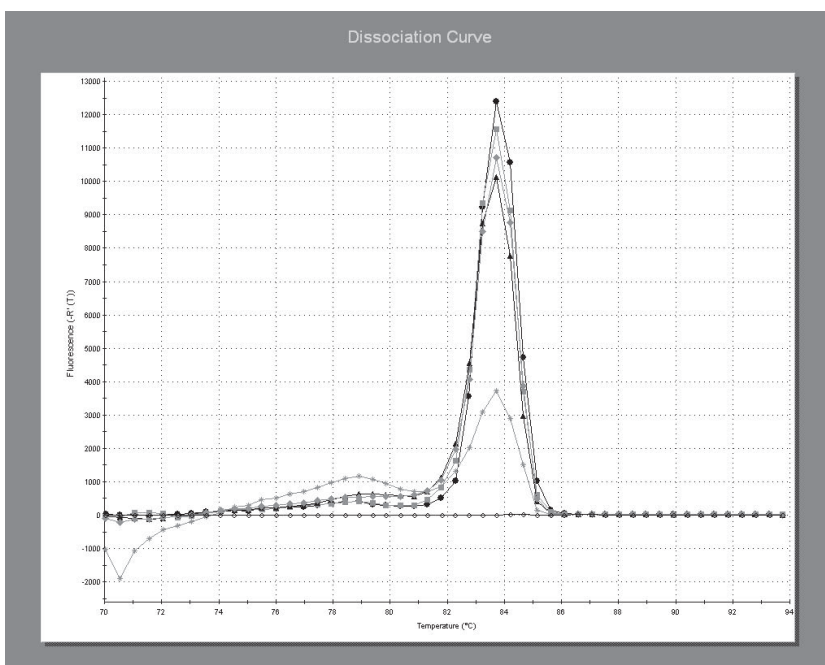
a)

b)

Fig. 2. Dissociation curves for amplicons obtained in real time RT-PCR reaction for PepMV-PK and PepMV-SW
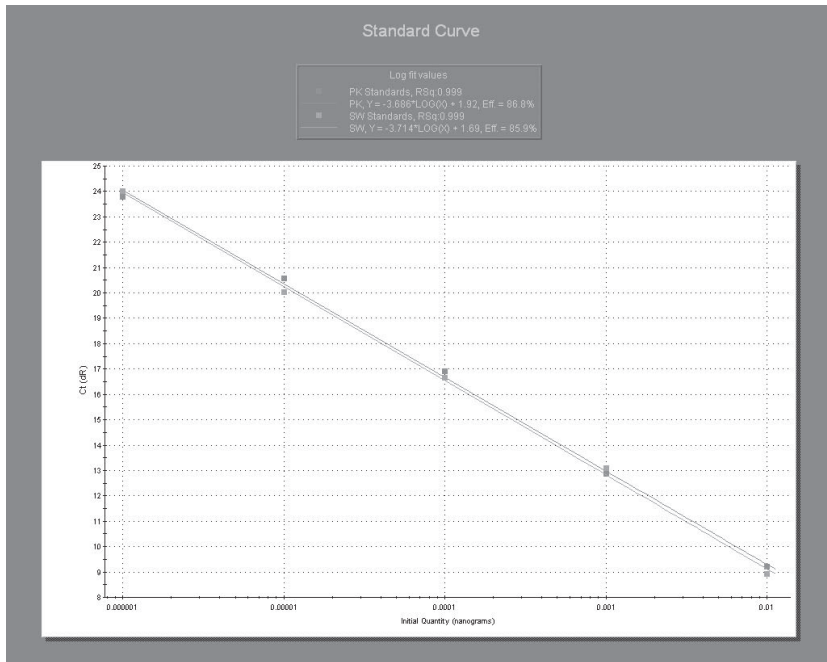
Fig. 3. Standard curves prepared with the primers F5 and R5 and 10-fold serial dilutions of PepMV-
-PK and PepMV-SW total RNA

genotype in plants infected with both isolates of both genotypes was observed in Belgian greenhouses (Hanssen *et al.* 2008). The increase in genome sequences diversity found in the field populations of PepMV presents a challenge for finding a conserved sequence region for primer development. In the present study, a conserved sequence region of 166 bp in coat protein gene was selected after evaluating a multiple alignment of the full genome sequences of 13 PepMV isolates. The primers were capable of detecting EU and Ch2 types collected in Poland. The real time RT-PCR described above is highly sensitive. It is capable of detecting about 100 fg of viral RNA in infected tomato and tobacco plants. The real time RT-PCR can be used when the virus concentration in infected plants is low and the standard test ELISA failed. We cannot only detect but also differentiate both genotypes by *T*m analysis. Melting curve analysis revealed distinct melting temperature peaks – for PepMV-PK range from 82,7–83,2°C and for PepMV-SW from 83,8–84,2°C. However, quantitation with dsDNA dyes is usually less accurate than sequence-specific probes because all dsDNA products are detected, including primer-dimers. During the optimization process the formation of non-specific amplification products was monitored using the dissociation curve analysis. A more practical application of real time RT-PCR for PepMV detection in samples is possible with immunocapture sample preparation (Ling *et al.* 2007). This assay combines the advantages from two widely used detection techniques: ELISA and PCR for plant viruses. We are also going to develop this method. The real time RT-PCR assay could serve as an excellent diagnostic and epidemiologic tool for PepMV detection in tomato and other hosts, as well as for the detection and confirmation of PepMV infection in tomato seeds.

ACKNOWLEDGEMENTS

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POLISH SUMMARY

WYKORZYSTANIE METODY REAL TIME RT-PCR DO DETEKCJI GENETYCZNIE ZRÓŻNICOWANYCH IZOLATÓW WIRUSA MOZAIKI PEPINO (*PEPINO MOSAIC VIRUS*)

Wirus mozaiki pepino (*Pepino mosaic virus*) – groźny patogen pomidora charakteryzujący się dużą zmiennością genetyczną mającą wpływ na jego diagnostykę. Na świecie zidentyfikowano kilka genotypów tego wirusa: EU, LP, US1,US2 i Ch2 oraz różnego typu rekombinanty. W Polsce stwierdzono obecność dwóch typów EU i Ch2. W celu skutecznej i szybkiej identyfikacji genetycznie różnych izolatów *Pepino mosaic virus* została wykorzystana technika real time RT-PCR. Uzyskane wyniki pozwoliły na wykrycie do 100 fg wirusowego RNA (2,71x10^3 kopii RNA) w zainfekowanych tkankach pomidora (*S. lycopersicum*) oraz tytoniu (*N. benthamiana*). Zaletą metody, obok dużej czułości i specyficzności, jest możliwość rozróżnienia genotypów PepMV analizując krzywe dysocjacji i temperatury topnienia. Metoda może być stosowana do detekcji wirusa występującego niekiedy w bardzo niskim stężeniu (na przykład w nasionach) oraz w sytuacjach gdy klasyczne testy typu ELISA lub RT-PCR nie dają jednoznacznych wyników.