DEVELOPMENT AND EVALUATION OF A MULTIPLEX RT-PCR FOR DETECTING MAIN VIRUSES AND A VIROID OF POTATO

M. PEIMAN[1,2], C. XIE[1*]

[1]Key Laboratory of Horticultural Plant Biology, Huazhong Agricultural University, Wuhan 430070, P.R. China; [2]Takestan Islamic Azad University, Shami shop, Talestan (Takestan), Iran

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Summary. – A multiplex RT-PCR (mRT-PCR) for detecting four potato viruses (Potato virus S (PVS), Potato virus X (PVX), Potato virus Y (PVY), and Potato leaf roll virus (PLRV)) and one potato viroid (Potato spindle tuber viroid (PSTVd)) was developed. The mRT-PCR consisted of one reaction with specific primers designed according to the sequences of coat protein (CP) genes of respective viruses and the sequence of the viroid. The entire procedure from tissue grinding to RT-PCR results takes about 4 hrs. The reliability of the method was tested on leaves sampled from the field, greenhouse and *in vitro*-grown plants by comparison with double-antibody sandwich ELISA (DAS-ELISA). A high correlation between these two methods was observed. The mRT-PCR was also evaluated by testing infected samples obtained from the International Potato Center, Lima, Peru; this testing confirmed its high reliability and sensitivity.

Key words: ELISA; multiplex RT-PCR; potato; viroid; virus

Introduction

China is the biggest potato (*Solanum tuberosum*) producer worldwide with planting area of 4.72 million hectares and production of about 64 million tones (Qu *et al*., 2004). However, the potato yield, in average about 16 tones per hectare (with a variation from 5 to 30 tones per hectare), is very low (Chen, 2004). One of the main reasons for this fact is a virus and/or viroid infection that usually cause crop losses. PVX (the family *Flexiviridae*, the genus *Potexvirus*), PVS (the genus *Carlavirus*), PLRV (the family *Luteoviridae*, the genus *Polerovirus*), PVY (the family *Potyviridae*, the genus *Potyvirus*), and PSTVd are regarded as the most common viruses and viroid, respectively, affecting the potato in many countries (Nie and Singh, 2001). The seed tolerance to this viroid is low. For instance, The Canadian Food Inspection Agency has reported a zero tolerance of tubers to PSTVd (De Boer *et al*., 2002). Therefore, the establishment of a virus-free seed source is one of the important measures for controlling potato virus diseases.

ELISA is currently used in potato seed certification programs, but it is time consuming and can only detect a specific virus with a specific serum in a single reaction (Shalaby and Mazyad, 2002). The past decade has experienced important development in molecular diagnostic techniques (Ellis and Zambon, 2002). Among them, RT-PCR provides sensitive detection of specific viral RNA sequences (Nicolaisen *et al.*, 2001) and has been adapted to identify various pathogen isolates (Henson and French, 1993) and monitor PLRV in both plants and aphids (Singh, 1998). More recently, a multiplex PCR has been employed for identifying viruses in many crops and trees (Nassuth *et al.*, 2000; Singh *et al*., 2000). To differentiate the strains NA-PVY^{NTN} from NA-PNY^{N}, a competitive RT-PCR was developed (Nie and Singh, 2002). For detecting potexviruses a single primer pair was used in RT-PCR (van der Vlugt and Berendsen, 2002).

*Corresponding author. E-mail: Xiech@mail.hzau.edu.cn; fax: +8627-87396057.

Abbreviations: CP = coat protein; DAS-ELISA = double-antibody sandwich ELISA; mRT-PCR = multiplex RT-PCR; PLRV = Potato leaf roll virus; PSTVd = Potato spindle tuber viroid; PVS = Potato virus S; PVX = Potato virus X; PVY = Potato virus Y

In this study, we developed a mRT-PCR for detecting several viruses and a viroid, all infecting the potato, in a short period. Its reliability was evaluated by testing potato samples from different sources and by comparing the results with those obtained by DAS-ELISA.

Materials and Methods

Plant material. Potato plants positive for PVX, PLRV, and PVS, and tobacco plants positive for PVY were provided by the Potato Germplasm Centre of China, Potato Research Institute, Helongjiang Academy of Agricultural Sciences, Harbin, P.R. China. PSTVd was collected from the potato field in Enshi, Hubei Province, P.R. China. Seventy-one leaf samples (with and without infection symptoms) originated from a potato field (11), greenhouse (12) and *in vitro* plantlets (20) from Wuhan, P.R. China, and different fields located at various altitudes in Qi Li Ping (500 masl), Tian Chi Shan (1100 masl) and Chang Ling Gang (1640 masl), Enshi, Hubei Province (28).

Total RNA from potato leaves was extracted as follows. About 1 ml of a buffer (0.1 mol/l NaCl; 2% SDS; 50 mmol/l Tris-HCl pH 9, and 10 mmol/l EDTA) was added to 300 mg of leaf tissue in a plastic bag and the mixture was kept at room temperature for 10 mins. Then 200 µl of phenol and 200 µl of chloroform was added and, after shaking for 3 mins, the suspension was centrifuged in a microcentrifuge tube for 5 mins. The aqueous upper phase was saved and ethanol-precipitated. RNA was finally dissolved in 25 µl of nuclease-free water per pellet.

mRT-PCR. To synthesize cDNAs, a mixture consisting of 50 pmoles (1 µl) of a random primer (6~9mer), 1 µg of total RNA, 2.5 mmol/l (8 µl) dNTPs, 20 U (0.5 µl) of an RNase inhibitor, and 1 µl of AMV reverse transcriptase (Takara Biotechnology) was incubated first at room temperature for 10 mins and then at 42°C for 1 hr; finally, the reaction was stopped by chilling on ice for 2 mins. The cDNAs were amplified by PCR in a UNO-Thermoblock (version 3.30) in a 20 µl volume containing 2 µl of 10×PCR buffer, 1.5 µl of 25 mmol/l $MgCl_2$, 2 µl of 2 mmol/l dNTPs, 0.2 µl of Taq DNA polymerase (Shanghai Promega), and 1 µl of cDNA. Whereas in classical RT-PCR 1 µl of each of the primer was used in each of several reactions, in mRT-PCR, different amounts of dNTPs (1 µl and 2 µl) and primers (0.3, 0.5 and 1 µl of 10 µmol/l solution) were tested in a single reaction. The PCR cycling consisted of 94°C/1.5 min (initial denaturation), 38 cycles of 94°C/45 secs (denaturation), 53°C/45 secs (primer annealing) and 72°C/1 min (extension), followed by final extension at 72°C for 5 mins. 10 µl of the products was analyzed by electrophoresis in a 2% agarose gel.

DAS-ELISA was carried out using antisera kindly provided by Dr. L.F. Salazar, International Potato Center, Lima, Peru, and the method described by Clark and Adams (1997). All samples were tested by ELISA with four repetitions.

Results and Discussion

Design of primers for mRT-PCR

The presence of different degenerate primers in a single reaction mixture in mRT-PCR can cause significant difficulties (Sharman *et al*., 2000). Designing primers for a mRT-PCR requires consideration of primer specificity, primer melting temperature (T_m) compatibility, and amplicon size (Singh and Nie, 2003). Also, the theoretical optimal and maximum annealing temperatures of the primer pairs should be kept to minimum. In this study, the primers were designed specifically for PVX, PVS, PVY and PLRV based on the alignment of nucleotide sequences of CP of more than 20 different isolates deposited in the GenBank. The primers for PSTVd were designed in a similar manner. Multiple sequence alignment was done by CLUSTAL W (version 1.82) and the best conserved regions were selected as primer targets. The properties of the primers were analyzed by the Primer Premier software (version 5.00, Premier Biosoft International) to allow accurate (T_m) calculation. The primers are given in Table 1.All the five selected primer pairs amplified their targets specifically (Fig. 1).

Table 1. The primers used for the detection of PVY, PVS, PLRV, PVX, and PSTVd

Virus/viroid	Primer	Primer sequence (5'-3')	Position of the amplicon (nts)	Acc. No.[a]
PVY	Forward	TGTGATGAATGGGCTTATG	540–721 (CP gene)	AF522296
	Reverse	TCTGCAACATCTGAGAAA		
PVS	Forward	GCTGTTYARATGGAAATCC	316–591(CP gene)	AF493951
	Reverse	CRTACARCCTRCACACYTT		
PSTVd	Forward	AAACTCGTGGTTCCTGTGG	8–345	AY5328804
	Reverse	CGGTTCCAAGGGCTAAAC		
PLRV	Forward	GGAAATGTCAATGGT	21–412 (CP gene)	X77321
	Reverse	GGGGTCCAACTCATAAGC		
PVX	Forward	YACTGCAGGCGCAACTCC	59–624 (CP gene)	Z23256
	Reverse	GTCGTTGGATTGYGCCCT		

Y = C or T; R = G or C.

[a]The sequence used for designing the primers.

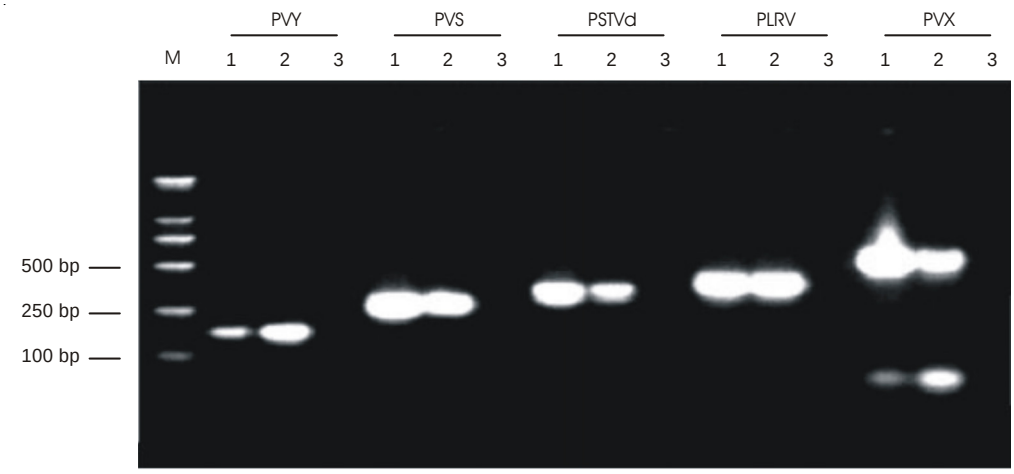

Fig. 1

mRT-PCR products of PVX, PLRV, PSTVd, PVS and PVY

Agarose gel electrophoresis. Individual viruses and viroid (lanes 1), mixture of all the tested viruses and viroid (lanes 2); uninfected control (lanes 3). DNA size marker (lane M).

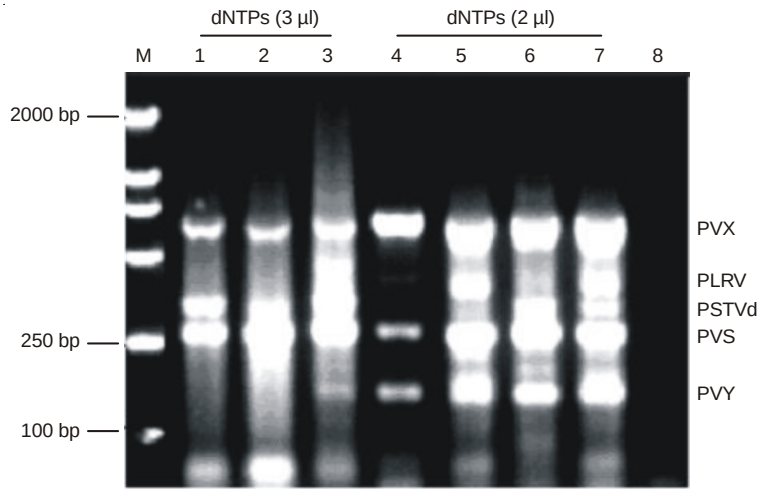

Fig. 2

mRT-PCR products amplified with different amount of dNTPs and primers

Agarose gel electrophoresis. a mixture of all the tested viruses and viroid with 0.3 µl of each of the primers (lanes 1 and 5); a mixture of all the tested viruses and viroid with 0.5 µl of each of the primers (lanes 2 and 6); a mixture of all the tested viruses and viroid with 1 µl of each of the primers (lanes 3 and 4); a mixture of all the tested viruses and viroid with 0.3 µl of each of the primers for PVS, PVX and PLRV or 0.5 µl of each of the primers for PVY and PSTVd (lane 7); uninfected control (lane 8); DNA size marker (lane M).

Optimization of mRT-PCR

Transition from a classical one-primer-pair RT-PCR to mRT-PCR, especially their PCR step, requires reoptimization of reagent concentrations (Singh and Nie, 2003). It has been reported that the concentrations of Mg^{2+}, dNTPs and primers significantly affect the outcome in mRT-PCR (Edwards and Gibbs, 1995). In this study, various amounts of primers and dNTPs were tested to obtain sufficiently distinct and strong bands (Fig. 2). The results showed that a combination of 2 µl of 2 mmol/l dNTPs and 0.3 µl of each of the primers for PVS, PVX and PLRV or 0.5 µl of each of the primers for PVY and PSTVd (all 10 µmol/l) was optimal. Summing up, the developed mRT-PCR allowed a specific and sensitive

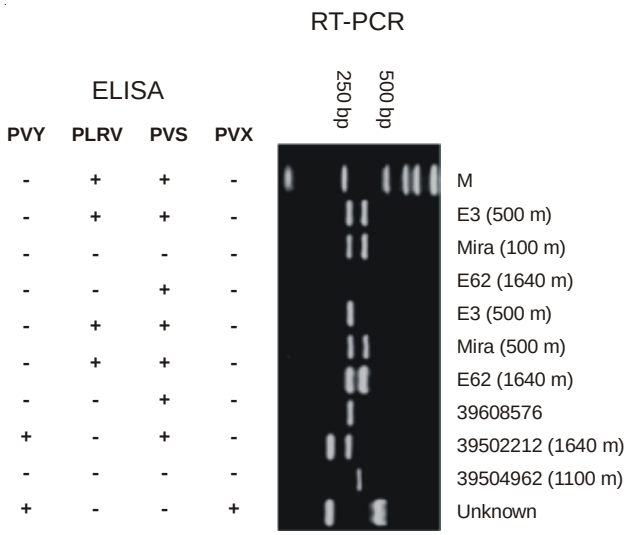

Fig. 3

Testing the reliability of mRT-PCR by comparison with ELISA

Ten potato leaf samples originating from different locations in China were tested by ELISA for PVY, PLRV, PVS and PVX, and by mRT-PCR for PVY, PLRV, PVS, PVX and PSTVd. ELISA results were evaluated as positive (+) or negative (-). DNA size marker (lane M).

detection of PVX, PVY, PLRV, PVS and PSTVd in a single reaction of PCR, a performance so far unattainable by ELISA.

Detection of viruses and a viroid of potato by mRT-PCR

The mRT-PCR produced with PVY, PVS, PSTVd, PLRV and PVX specific bands of 181, 275, 337, 391 and 565 bp, respectively (Fig. 1). These results demonstrated a successful use of mRT-PCR for detecting four viruses and one viroid in a single reaction. Previous researches have indicated that, in a duplex RT-PCR, the use of a random primer in cDNA synthesis leads to a better detection of PLRV and PVY as compared with specific primers (Nie and Singh, 2001) and a better amplification of shorter fragments as compared to longer ones (Singh and Singh, 1997). However, we could not confirm these findings, as a shorter fragment (181 bp) was amplified as well as a longer one (565 bp) (Fig. 2). Nevertheless, in general, the literature data show that an uneven amplification of amplicons of different size still represents a common problem. In order to reduce a non-specific amplifications and to increase the specific one, the intractions among different primers should be kept to minimum (Nie and Singh, 2001) and the quality and quantity of cDNA used in the PCR step should be taken in consideration (Singh *et al*., 2000).

A rapid protocol for detecting potato viruses in aphids or potatoes by mRT-PCR has been described by Singh *et al.* (2004); however, it appears more time-consuming than our procedure (4 hrs), as it is supposed to take 6 hrs.

Reliability of mRT-PCR

Ninety-nine samples, collected from different regions of China, were subjected to both mRT-PCR and DAS-ELISA with four repetitions. The results showed consistence between these two methods, indicating high reliability of the developed mRT-PCR (Fig. 3). Furthermore, potato plant samples positive for PVX, PLRV and PVS separately and tobacco plant samples positive for PVY were tested by classical RT-PCR and mRT-PCR. These comparative tests gave consistent results (data not shown). All these results indicate that the developed mRT-PCR is a useful tool for rapid, simple and reliable detection of several viruses and a viroid of the potato. The mRT-PCR has a capacity to handle a great number of samples in a short time of about 4 hrs and its results are comparable to those of widely used ELISA. Moreover, this mRT-PCR appears a low running cost method that offers a potential for potato tuber examination for viruses (viroids). Ther latter may largely reduce the expenses of growing plants in a protected condition, which is needed by other virus detection methods.

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References

Clark MF, Adams AN (1977): Characteristic of the microplate method of enzyme-linked immunosorbent assay for detection of plant viruses. *J. Gen. Virol.* **34**, 475–483.

Chen M (2004): The present and prospect of potato industrial development in China. *Proc. V. Int. Conf. WPC*. China, pp. 82–83.

De Boer SH, Xu H, DeHaan TL (2002): Potato spindle tuber viroid not found in western Canadian provinces. *Plant Pathol.* **24**, 372–375.

Edwards MC, Gibbs RA (1995): Multiplex PCR. In Diefenbach CW, Dveksler GS (Eds): *PCR Prime. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, pp. 157–171.

Ellis JS, Zambon MC (2002): Molecular diagnosis of influenza. *Rev. Med. Virol.* **12**(6), 375–389.

Henson JM, French R (1993): The polymerase chain reaction and plant disease diagnosis. *Annu. Rev. Phytopathol.* **31**, 81–109.

Nassuth A, Pollari E, Helmeczy K, Stewart S, Kofalvi SA (2000): Improved RNA extraction and one-tube RT-PCR assay for simultaneous detection of control plant RNA plus several viruses in plant extracts. *J. Virol. Methods* **90**, 37–49.

Nicolaisen M, Rasmussen HN, Husted K, Nielsen SL (2001): Reverse transcription-detection of immobilized, amplified product in a one-phase system (RT-Diapops) for the detection of potato virus Y. *Plant Pathol.* **50**, 124-129.

Nie X, Singh RP (2002): Probable geographical grouping of PVY^{N} and PVY^{NTN} based on sequence variation in P_1 and 5′UTR of PVY genome and methods for differentiation North American PVY^{NTN}. *J. Virol. Methods* **103**, 145–156.

Nie X, Singh R (2001): A novel usage of random primers for multiplex RT-PCR detection of virus and viroid aphids, leaves and tuber. *J. Virol. Methods* **91**, 37–49.

Qu D, Xie K, Jin L, Bian C, Duan, S (2004): Development of potato industry and technology needs in China. *Proc. V. Int. Conf. WPC*. China, pp. 84–87.

Shalaby A, Mazyad H (2002): Virus detection: PVY, PVX and PLRV multiplex-RT-PCR. *Technical Sheet* No 12.

Sharman M, Thomas JE, Dietzgen RG (2000): Development of a multiplex immunocapture PCR with colourimetric detection for viruses of banana. *J. Virol. Methods* **75**, 75–88.

Singh RP, Dilworth AD, Singh M, McLaren DL (2004): Evaluation of a simple membrane-based nucleic acid preparation protocol for RT-PCR detection of potato viruses from aphid and plant tissuses. *J. Virol. Methods* **121**, 163–170.

Singh RP (1998): Reverse transcription polymerase chain reaction for the detection of viruses from plants and aphids. *J. Virol. Methods* **74**, 125–138.

Singh RP, Nie X (2003): Multiplex virus and viroid detection and strain separation via multiplex reverse transcription polymerase chain reaction. *Can. J. Plant Pathol.* **25**, 127–134.

Singh RP, Nie X, Singh M (2000): Duplex RT-PCR: reagent concentrations at reverse transcription stage affect the PCR performance. *J. Virol. Methods* **86**, 121–129.

Singh M, Singh RP (1997): Potato virus Y detection: sensitivity of RT-PCR depends on the size of fragment amplified. *Can. J. Plant Pathol.* **19**, 149–155.

van der Vlugt RAA, Berendsen M (2002): Development of general Potexvirus detection method. *Eur. J. Plant Pathol.* **108**, 367–371.