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Note

Development of Single-tube Multiplex Immunocapture RT-PCR Assay for Simultaneous Detection of Two Pepper Tobamoviruses

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An immunocapture reverse transcription-polymerase chain reaction (IC/RT-PCR) was developed for simultaneous detection of two pepper-infecting RNA viruses, *Pepper mild mottle virus* (PMMoV) and *Tobacco mild green mosaic virus* (TMGMV). The assay could be performed in a single tube for simultaneous and sensitive detection of these tobamoviruses. This detection system revealed thousand-fold increase in detection sensitivity compare to ELISA. This method could save time and reagent cost compare to common RT-PCR which needs several reactions and several procedures of viral RNA extractions for the same number of samples.

Keywords : Detection, ELISA, IC/RT-PCR, pepper, PMMoV, TMGMV, Tobamovirus

Under field conditions, pepper (*Capsicum annuum* L.) are often infected by several viruses during an entire growing season (Green and Kim, 1991; Kang, 1973), resulting in decreased yield and quality of fruits. The common viruses affecting pepper crops singly or in combination, in Korea include *Broad bean wilt virus* (BBWV), *Cucumber mosaic virus* (CMV), *Pepper mottle virus* (PepMoV), *Pepper mild mottle virus* (PMMoV), *Tobacco mild green mosaic virus* (TMGMV), *Tomato spotted wilt virus* (TSWV) (Choi et al., 2005).

PMMoV and TMGMV, which belong to the genus Tobamovirus, are the most significant pathogenic causes of economic loss in pepper production worldwide (Cohen et al., 2002; Gibb, 1988). Typically, PMMoV and TMGMV are transmitted through seed and soil (Komuro and Iwaki, 1969; Lanter et al., 1982). Once a pepper field is contaminated with the virus, decontamination is extremely difficult as long as pepper is continuously cultivated; the virus persists stably in infected plants and infects newly transplanted seedlings.

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The determination of virus infection in plants using serological or biochemical techniques is important for the prevention of virus diseases. Serological techniques such as ELISA are more convenient and inexpensive than existing technique, although they require some specialized equipment. However, cross reaction between tobamoviruses and their antisera (Jacobi and Castello, 1991) limits serological differentiation of theses viruses by ELISA. The technique is therefore unsuitable for detecting mixed infections in tissues and monitoring viruses separately in pepper field.

As such many reverse transcription polymerase chain reaction (RT-PCR) protocols have been developed to individual viruses from crop plants (Grotzinger and Will, 1992; Pappu et al., 1993). However, detection of several individual viruses separately by RT-PCR is expensive. To reduce the cost, a duplex RT-PCR has been developed (Osiowy, 1998; Rosenfield and Jaykus, 1999; Nassuth et al., 2000). Recently, immunocapture reverse transcriptase-polymerase chain reaction (IC/RT-PCR) assays have been developed for the detection of several economically important viruses (Nolasco et al., 1993; 1998; Jacobi et al., 1998; Sharman et al., 2000).

In this study, we have developed a multiplex IC/RT-PCR for the simultaneous detection of both PMMoV and TMGMV and compared the sensitivity and specificity of the IC/RT-PCR approach with that of ELISA systems currently in use.

PMMoV and TMGMV were isolated from red pepper. After three repetition of single local lesion isolation on *Nicotiana glutinosa*, the biologically pure isolate were maintained in *N. occidentalis*. For this study, single and mixed infection with PMMoV and TMGMV were conducted with carborundum-dusted *Capsicum annuum* 'Eary Calwonder' in a green house at 25±2°C. Test samples consisted of two types: (i) crude extracts prepared from fresh tissue, and (ii) dilutions of purified virus. The extract for ELISA and IC/RT-PCR, 0.05-0.1 g of pepper tissues were extracted in ELISA extraction buffer containing 0.2% bovine serum albumin (PBS-TPB) to give a final dilution of

1:10. For direct comparison of detection sensitivity both ELISA and IC/RT-PCR, purified viruses were diluted in PBS-TPB to concentrations ranging from 10 μg/ml to 100 fg/ml.

DAS-ELISA was carried out mainly as described by Clark and Adams (1977). Microtiter plates (Polystyrene plates, Costar) were coated for 3h at 37°C with 100 μl per well of IgG diluted to 1 mg/ml in coating buffer (pH 9.6). The plates were then washed three times for 3 mins with PBS-T. About 150 μl of plant extract were added to each well and the plates were incubated over night at 4°C. Plates were then washed three times with PBS-T and incubates for 3h at 37°C with 100 μl per well of IgG-enzyme conjugate. After washing the plates, as described previously, 100 ml of substrate (1 mg/ml of p-nitrophenil phosphate in 10% diethanolamine, pH 9.8) was added to each well. Absorbance was read at 405 nm in a microtiter plate reader.

Immunocapture (IC) was carried out using a coating and trapping method adapted from Clack and Adams (1977). Micro tubes (200 μl) were coated with antibodies (50 μl) to both PMMoV and TMGMV at concentrations of 1 mg/ml each. The tubes were then incubated 2 hr at 37°C. After washing the tubes three times with PBS-T, 50 μl of sample (crude extracted sample or dilution of purified virus) was added per tube, and incubated overnight at 4°C or 2-3 hrs at 37°C. Samples were removed by pipette, 80 μl/tube of PBS-T added, spun as above and wash solution was pipetted off. This PSB-T washing step was repeated once, and followed by a final wash with deionized water.

RT-PCR was performed using an 'AccessQuick RT-PCR' kit (promega). RT-PCR reaction mix was added to micro tubes, after washing. The reaction mix contained: 12.5 μl matermixture; 0.5 μl AMV reverse transcriptase; 1 μl (10 pmole) each primer and nuclease free distilled water up to final volume of 25 μl. The primer sequences were based on the published sequence of genomes of PMMoV-Kr (Yoon, 2003) and TMGMV-KP (Choi et al., 2002). The primers used for the specific detection of PMMoV and TMGMV were PM317-F 5'-ACA GTT TCC AGT GCC AAT CA-3', PM317-R 5'-AAG CGT CTC GGC AGT TG-3' (flanking a 317 base position of the coat protein of PMMoV), and CPTMG-F 5'-TCG AGT ACG TTT TAA TCA AT-3' and CPTMG-R 5'-ATT TTA GGA AAT CTC ACA AC-3' (flanking a 510 base position of the coat protein of TMGMV). Tubes were spun briefly, reverse transcriptase (RT) reaction was carried out with on cycle at 42°C for 45 mins and 35 cycles of PCR amplication using the step program (95°C, 45 sec.; 50°C, 50 sec. and 72°C, 60 sec.), followed by final extension at 72°C for 10 mins (Choi et al., 2002).

This study was conducted to develop an IC/RT-PCR assay for simultaneous detection of PMMoV and TMGMV in pepper that would improve both detection sensitivity and specificity compare to the ELISA systems currently in use. A duplex IC/RT-PCR assay successfully detected simultaneously two different tobamoviruses. Two RT-PCR products corresponding to PMMoV (317bp) and TMGMV (510 bp), could be differentiated by their size on the same lane (Fig. 1, Lane 3). As expected, no virus was detected in ether buffer or healthy sap negative control. The IC/RT-PCR approach was chosen rather than hybridization or RT-PCR tests because of its high sensitivity due to the initial immunocapture enrichment step followed by PCR amplification; high specificity due to the combination of virus specific antibody capture and primer specificity; and virus detection directly in crude plant extracts rather than in partially or highly purified nucleic acid preparations. Studies on the detection of economically important fruit tree viruses have documented the potential of IC/RT-PCR to overcome limitations for virus detection in crude tissue extracts of woody plants (Candresse et al., 1995; Jacobi et al., 1997; Wetzel et al., 1992).

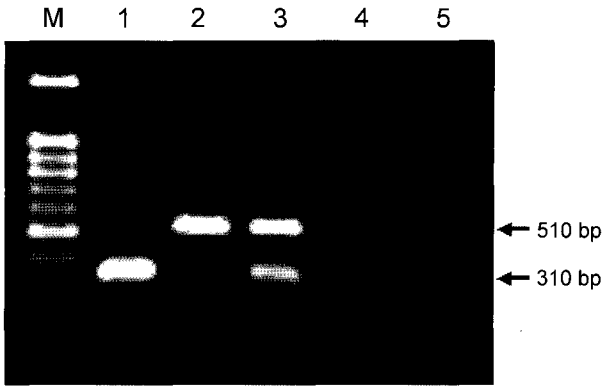

Fig. 1. Amplication and differentiation of PMMoV and TMGMV by duplex IC/RT-PCR. All tubes were coated with purified Immunoglobulin G to both viruses, and virus from infected plant sap were captured and RT-PCR reactions were performed with primer pairs PM317-F/-R and CPTMG-F/-R. Lane 1, PMMoV; 2, TMGMV; 3, PMMoV+TMGMV; 4, Buffer and 5, Healthy control. M, 100bp ladder.

A direct comparison of detection sensitivitiy with ELISA in the presence of both viruses was not possible as the ELISA assay could not reliably distinguish between PMMoV and TMGMV. The IC/RT-PCR detection limit was 1 pg/ml or 10 pg/ml of either PMMoV or TMGMV (Fig. 2). These results compare well with those reported for other plant viruses. The IC/RT-PCR detection limit for *Apple chlorotic leaf spot virus* (ACLSV) and *Plum pox virus* (PPV) were 0.1 pg/ml (Candresse et al., 1995). Detection limits for *Prune dwarf virus* (PDV), *Prunnus necrotic ringspot virus* (PNRSV), *Grape vein fan leaf virus* (GFLV), and *Cherry leaf roll vrius* (CLRV) ranged from

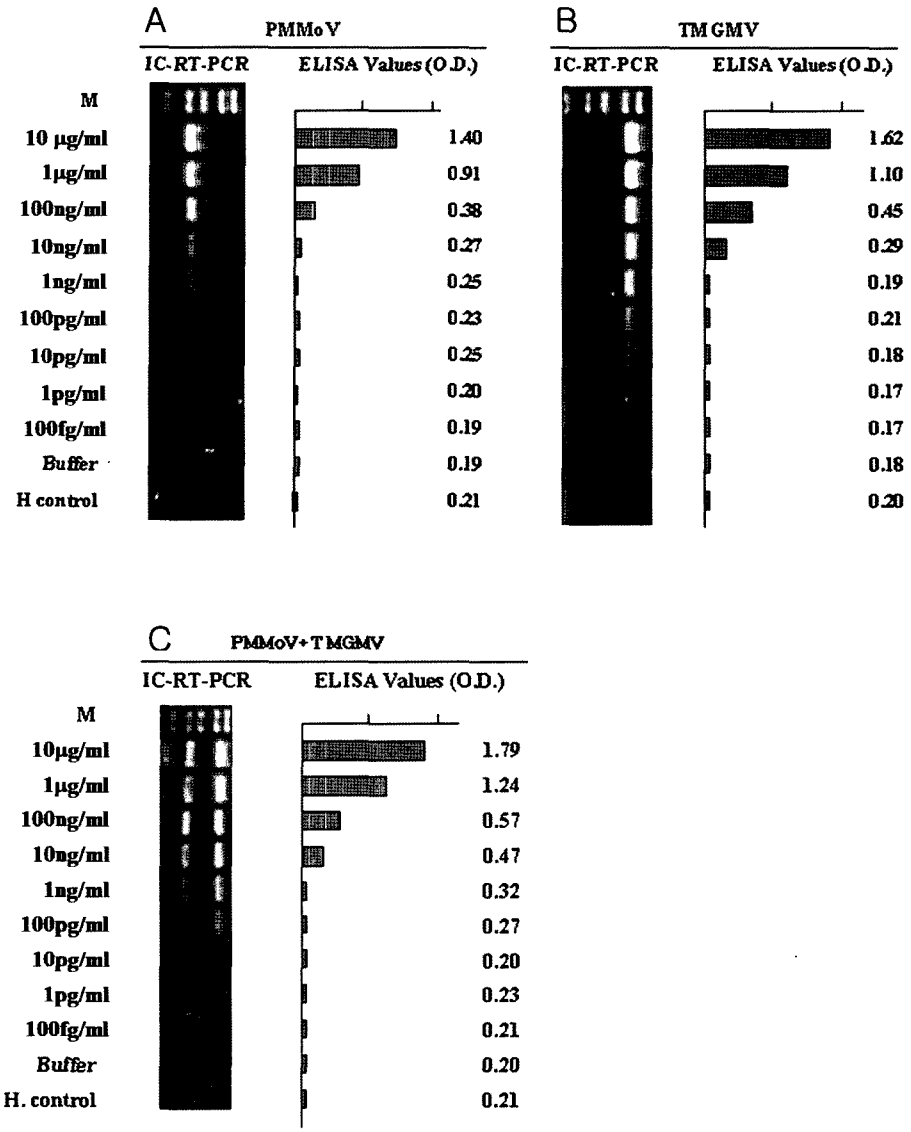

Fig. 2. Sensitivity and specificity of duplex IC/RT-PCR for detection of PMMoV and TMGMV. All tubes were coated with purified Immunoglobulin G to both viruses and all RT-PCR reactions were conducted with primer pairs PM317-F/-R and CPTMG-F/-R. Healthy tobacco plant extract diluted at 10^{-2} in PBS-TPB served as diluent for purified PMMoV (A), purified TMGMV (B), or both viruses. PMMoV, TMGMV, or both viruses at final concentrations ranging from 10 μg/ml to 100 fg/ml.

0.2 to 2 pg/ml (Rowhani et al., 1995). Due to the lack of quantitative data on multiplex IC/RT-PCR detection of other plant viruses, we could not contrast our results with those of others.

In contrast with ELISA, which could not discriminate between PMMoV and TMGMV, the experiments confirmed the specificity of primer pairs PM317-F/-R and CPTMG-F/-R for their respective target viruses in both single-virus and duplex IC/RT-PCR assays. Based on this finding, the duplex system was used for survey of virus infecting pepper plants.

Traditional methods used to confirm the presence of both viruses include agarose gel double diffusion analysis, or inoculation of two herbaceous indicator plant species, each producing symptoms only in response to infection by either PMMoV or TMGMV even in the presence of both viruses. Gel diffusion tests require substantial amounts of virus and antiserum, which may not always be available. In case of indicator plants tests, it takes between 3-7 days for symptoms development, whereas IC/RT-PCR can be performed in 1 day, including gel analysis.

The duplex IC/RT-PCR assay for detection and differentiation of PMMoV and TMGMV should be useful in the following application; test on field pepper plants to monitor

virus distribution and host range and test on virus-inoculated greenhouse seedlings to study infection mechanisms and virus movement. Its high sensitivity may helpful to eliminate the need for sample purification and concentration prior to virus detection.

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References

Candresse, T., Lanneau, M., Revers, F., Macquaire, G., German, S., Dunez, J., Grasseau, N. and Malinovsky, T. 1995a. An immunocapture PCR assay adapted to the detection and the analysis of the morecular variability of *Apple chlorotic leaf spot virus*. *Acta Hort.* 386:136-147.

Choi, G. S., Kim, J. H., Lee, D. H., Kim, J. S. and Ryu, K. H. 2005. Occurrence and distribution of viruses infecting pepper in Korea. *Plant Pathol. J.* 21:258-261.

Choi, G. S., Kim, J. H., Ryu, K. H., Choi, J. K., Chae, S. Y., Kim, J. S., Chung, B. N., Kim, H. R. and Choi, Y. M. 2002. Frist report of *Tobacco mild green mosaic virus* infecting pepper in Korea. *Plant Pathol. J.* 18: 323-327.

Clark, M. F. and Adams, N. 1977. Characteristics of the microplate method of enzyme-linked immunoassay (ELISA) for the detection of plant viruses. *J. Gen. Virol.* 34:475-483.

Cohen, J., Rosner, A. Kagan, S. Lampel, S., Beckelman, H., Maslenin, L., Zeidan, M. and Gera, A. 2002. A new disease in Tabernamontana caused by *Tobacco mild green mosaic virus*. *Acta Hort.* 569:103-109.

Gibb, A. J. 1988. Tobacco mild green mosaic virus. *Plant Viruses Online: Description and Lists from the VIDE Database.*

Green, S. K. and Kim, J. S. 1991. Characteristics and control of viruses infecting peppers: a literature review. *AVRDC Technical Bull.* No. 18:60.

Grotzinger, T. and Will, H. 1992. Sensitive methods for identification of minor hepatitis B mutant viruses. *Virology* 187:383-387.

Jacobi, V. and Castello, J. D. 1991. Isolation of tomato mosaic virus from water draning forest stands in New York State. *Phytopathology* 81:1112-1117.

Jacobi, V., Bachand, G. D., Hamelin, R. C. and Castello, J. D. 1997. Development of immunocapture PCR assay for tobacco and *Tomato mosaic tobamoviruses*. *Phytopathology* 87:S46 (Abstract).

Jacobi, V., Bachand, G. D., Hamelin, R. C. and Castello, J. D. 1998. Development of multiplex immunocapture RT-PCR assay for detection and differentiation of *Tobacco mosaic tobamoviruses*. *J. Virol. Methods* 74:167-178.

Kang, K. Y., Choi, J. I. and La, Y. J. 1973. Isolation and identification of virus affecting pepper in Korea. *J. Kor. Soc. Horti. Sci.* 13:35-44.

Komuro, Y. and Iwaki, M. 1969. Presence of *Tobacco mosaic virus* in roots of field-grown tomato plants healthy in appearance. *Ann. Phytopathol. Soc. Jpn.* 35:294-298.

Lanter, J. M., McGuire, J. M. and Goode, M. J. 1982. Presistence of *Tomato mosaic virus* in tomato debris and soil under field conditions. *Plant Dis.* 66:552-555.

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

Nolasco, G., de Blas, C., torres, V. and Ponz, F. 1993. A methods combining immunocapture and PCR amplification in a microtiter plat for the detection of plant viruses and subviral pathogens. *J. Virol. Methods* 45:201-218.

Osiowy, C. 1998. Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimes by a multiplex reverse transcription-PCR assay. *J. Clin. Microbiol.* 36:3149-3154.

Pappu, S. S., Brand, R., Papuu, H. R., Rybicki, E. P., Gough, K. H., Frenkel, M. J. and Niblett, C. L. 1993. A polymerase chain reaction methos adapted for selective amplification and cloning of 3' sequences of potyviral genomes: application to *Dasheen mosaic virus*. *J. Virol. Methods* 41:9-20.

Rosenfield, S. I. and Jaykus, L. A. 1999. A multiplex reverse transcription polymerase chain reaction method for the detection of foodborne viruses. *J. Food Prot.* 62:1210-1214.

Rowhani, A., Maningas, M. A., Lile, L. S., Daubert, S. D. and Golino, D. A. 1995. Development of a detection system for viruses of woody plants based on PCR analysis of immobilized virions. *Phytopathology* 85:347-352.

Sharman, M., Thomas, J. E. and Dietzgen, R. G. 2000. Development of a multiplex immunocapture PCR with colourimetric detection for viruses of banana. *J. Virol. Methods* 89:75-88.

Wetzel, T., Candress, T., Macquaire, G., Ravelonandro, M. and Dunez, J. 1992. A highly sensitive immunocapture polymerase chain reaction method for *Plum pox potyvirus* detection. *J. Virol. Methods* 39:27-37.

Yoon, J. Y. 2003. Molecular characterization of symptom attenuation of *Pepper mild mottle virus*. Ph.D. Thesis of Seoul Women's University, Korea.