CHEMICAL DEGRADATION OF TOBACCO MOSAIC VIRUS FOLLOWED BY INFECTIVITY ASSAY, REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION AND GEL ELECTROPHORESIS

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Summary. – In order to determine the detection limit for chemically treated virions by gel electrophoresis, reverse transcription–polymerase chain reaction (RT-PCR) and infectivity assay, tobacco mosaic virus (TMV) exposed to various concentrations of chemicals was studied. When virions were exposed to 0.2 N HCl for 30 mins, partially degraded TMV particles were observed by gel electrophoresis. Under the same exposure, a major RT-PCR amplified DNA product corresponding to the target size of 806 bp, which decreased as a function of time, could be detected for up to 60 mins of exposure. When virions were treated with NaOH (0.02 N or higher normality) for 5 mins, partially degraded virions were detected by gel electrophoresis, exhibiting multiple band patterns. Exposure of the virions to 0.1 N NaOH for 5 mins revealed severely degraded viral RNA, but disappearance of the amplified RT-PCR products was apparent during 30–60 mins of exposure. Therefore, these data showed clearly the difference in the detection limit of gel electrophoresis and that of RT-PCR for the degraded viral RNA. In addition, the infectivity assay showed that the number of local lesions in *Nicotiana rustica* were significantly reduced by more than 95% when the virus was exposed to 0.2 N HCl for 15 mins or 0.1 N NaOH for 10 mins. From these results we conclude that loss of infectivity was not related to that of PCR product. Other chemical disinfectants such as phenol or formalin were also found to be effective to reduce the virus infectivity, but a corresponding degradation of viral RNA was detected by neither gel electrophoresis nor RT-PCR.

Key words: tobacco mosaic virus; chemical degradation; UV irradiation; infectivity; RT-PCR; gel electrophoresis

Introduction

Molecular techniques such as PCR (RT-PCR) and nucleic acid hybridization have been used to detect viruses in various environmental specimens. However, there is a potential problem in that these methods are not able to differentiate infectious from non-infectious viruses (Ma *et al.*, 1994; Moore and Margolin, 1994). Chemical disinfectants have been reported to inactivate effectively either *in vitro* or *in vivo* a wide range of bacteriophages (Yahya *et al.*, 1992; Maillard *et al.*, 1994; Pesaro *et al.*, 1995), animal and plant viruses (Hu *et al.*, 1994; Ma *et al.*, 1994; Moore and Margolin, 1994; Rivas *et al.*, 1994). Although chemicals may disrupt viral coat proteins, causing the loss of viral infectivity, viral nucleic acids may not be destroyed completely by these disinfectants and may still be detected by molecular techniques. Therefore, many reliable assay techniques have been tested in order to evaluate the efficacy of virus inactivation. Gel electrophoresis has been

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Abbreviations: nt = nucleotide; PBS = phosphate-buffered saline; RT-PCR = reverse transcription–polymerase chain reaction; TMV = tobacco mosaic virus

demonstrated to be useful for detecting bacteriophages as well as several plant viruses including TMV in crude plant extracts, and to study their *in vitro* assembly products (Asselin and Grenier, 1985; McDaniel *et al.*, 1995; Serwer *et al.*, 1984).

Particularly, to monitor the population of infectious viruses, an infectivity assay is crucial.

TMV has worldwide distribution and causes a significant loss in many important crops including tobacco and tomato (Broadbent, 1976; Lanter *et al.*, 1982). This virus consists of a single-stranded RNA encapsidated with coat protein subunits. Since TMV is transmitted mechanically and remains infective for a year or more in soil or on tools, sanitation is one of the most important means to control the incidence and spread of TMV (Broadbent, 1976; Lanter *et al.*, 1982). In the present study, the treatment of TMV with various chemicals (disinfectants) and UV light was evaluated by gel electrophoresis, RT-PCR and infectivity assay.

Materials and Methods

Plant material and virus. N. rustica was transplanted into 15 × 5 cm pots after 2 weeks and grown in a greenhouse at a 16 hrs photoperiod for further 4 weeks. TMV, a Korean common strain (Koh *et al.*, 1992) was used. The virus was maintained and purified from *N. rustica* as described previously (McDaniel *et al.*, 1995).

Acidic and alkaline treatments. A TMV suspension at a final concentration of 50 µg/ml was mixed 1:1 with HCl or NaOH of various normality, and the mixture was incubated at 4°C for 0–120 mins. Aliquots (10 µl) were taken at selected time intervals, neutralized and adjusted to pH 7.2 with Hepes buffer (10 µl) (Sigma).

Formalin treatment. A 1:1 mixture of TMV in phosphate-buffered saline (PBS) pH 7.4 and 4% formalin (final concentration of formalin was 2%) was incubated at 4°C for 0–30 mins and then neutralized with 4% $Na_2S_2O_5$ in PBS.

Phenol treatment. A 1:1 mixture of TMV in PBS pH 7.4 and 4% phenol (final concentration of phenol was 2%) was incubated at 4°C for 0–30 mins and then neutralized with PBS.

UV irradiation was performed at 7 mW/cm² intensity for 30 mins.

RT-PCR assay. A set of primers (TMP2 and TMP1) based on the sequence described by Koh *et al.* (1992) was commercially synthesized by Bioneer Co., South Korea. TMP2 (5′-CGGTCAGTGCCGAACAAGAA-3′) corresponded to nucleotides (nt) 5,590–5,609 of TMV RNA. TMP1 (5′-TGGGCCCCTACCGGG-3′) was complementary to nt 6,381–6,395 of TMV RNA. The RT part of the assay was carried out at 42°C for 30 mins with 100 ng of acid- or base-treated TMV in 50 mmol/l Tris-HCl pH 8.3, 5 mmol/l $MgCl_2$, 75 mmol/l KCl, 1.0 mmol/l dNTPs, 50 pmoles of TMP1, 20 U of an RNase inhibitor (RNAguard, Pharmacia Biotech.), and 200 U of Moloney murine leukemia virus reverse transcriptase (Promega). The PCR part of the assay was done with the Bio-Rad Gene Cycler in a 100 µl volume containing 50 pmoles of each primer, 2.5 U of Taq DNA polymerase (Boehringer Mannheim), 200 µmol/l dNTPs, 10 mmol/l Tris-HCl pH 8.3, 1.5 mmol/l $MgCl_2$, and 50 mmol/l KCl. The amplification was initiated with a denaturation step at 95°C for 5 mins; this was followed by 40 cycles with the following denaturation, annealing and synthesis parameters: 94°C for 1 min, 46°C for 1 min, and 72°C for 1.5 min, respectively. An extension step at 72°C for 15 mins was included at the end of the amplification.

Gel electrophoresis of TMV RNA samples (30 µg) was performed in 1% agarose gel in a Tris-borate-EDTA buffer pH 8.3 at 100 V at 4°C. The gels were stained with ethidium bromide (1 µg/ml), visualized on a UV transilluminator, and photographed (Polaroid film type 667). A 1 kb DNA ladder (Gibco-BRL) or a λ DNA/*Hind*III digest was used as a size marker.

Infectivity assay. Each inoculum was diluted to 10 ml with 10 mmol/l Na_2HPO_4 pH 7.2 containing 1% celite as abrasive. Final concentration of TMV in the inoculum was 50 µg/ml. The inoculum was applied onto half-leaves of *N. rustica* with an artists' brush, alternating treatments on right and left halves. Assays were generally performed on uniform plants. To remove residual cytotoxicity of the chemicals tested, leaves were rinsed with tap water immediately after inoculation. Each sample was assayed at least in triplicate, usually in 6 replicates. Controls were made with non-treated TMV samples as well as mixtures of chemical/neutralizer without virus. Local lesions were counted 5 days after inoculation. For the calculation of the percentage of inhibition see the legend to Table 1.

Results and Discussion

We first attempted the agarose gel electrophoresis to test the degree of degradation of TMV particles in solutions

Table 1. Inactivation of TMV infectivity for *N. rustica* by various chemical disinfectants

Disinfectant (concentration or intensity)	Exposure time (mins)	Mean number of local lesions		Inhibition (%)
		Untreated TMV	Treated TMV	
HCl (0.2 N)	0	260	382	0
	5	266	50	81.2
	15	370	15	95.9
NaOH (0.1 N)	0	284	288	0
	10	365	10	97.3
UV (7 mW/cm²)	30	330	47	85.8
Phenol (2%)	0	273	201	26.4
	5	254	67	73.6
	30	223	22	90.1
Formalin (2%)	0	273	326	0
	5	290	77	73.4
	30	274	7	97.4

Inhibition = 100 – (A/B x 100) %. A = mean number of lesions for a treated sample. B = mean number of lesions for an untreated sample (control).

containing different concentrations of HCl or NaOH. When TMV was treated with these chemicals, gel electrophoresis showed a degradation of RNAs of TMV particles. As shown in Fig. 1, TMV treated with 0.2 N or 0.3 N HCl for 30 mins exhibited partially degraded TMV RNA bands, but no breakdown of viral RNAs was observed with 0.1 N HCl or HCl of lower normality. The susceptibility of TMV against NaOH was comparable. Results of our experiments in which TMV was exposed to 0.5 N NaOH for 5 mins revealed a completely undetectable viral RNA. When TMV was treated with 0.2 N NaOH for 5 mins, the degradation of virions was apparent and exhibited a different banding pattern similar to that of HCl-treated virus (Fig. 2). In addition, analysis by gel electrophoresis revealed that the TMV virions exposed to 0.1 N HCl for 30 mins remained structurally intact but those treated with 0.1 N NaOH for 5 mins were degraded completely. These results are not surprising since viral RNA is extremely sensitive to degradation by alkaline pH (Keeling and Matthews, 1982; Perham and Wilson, 1976, 1978; Wilson and Perham, 1985). The bands on the gel represent various, partially degraded forms (degradation intermediates) of TMV RNA. The existence of a series of intermediates in the alkaline disassembly of the virus has been previously identified and ascribed to the variability in the interaction between the RNA and the coat protein subunits along the TMV rod (Perham and Wilson, 1978).

Fig. 3 presents the intensity of the RT-PCR amplification of TMV treated with 0.2 N HCl for various time intervals. The intensity of the major (806 bp) band as well as of other minor bands was slightly lower and the bands were detected up to 60 mins of the treatment. With 0.3 N HCl, the intensity of the bands was distinctively lower and they could be detected up to 30 mins of the treatment, rendering no detectable bands after 60 mins of the treatment (Fig. 4). Obviously, these results showed that the intensity of detectable bands could be reduced by extending the exposure time at a given concentration of HCl.

When TMV was exposed to 0.1 N NaOH, the disappearance of the RT-PCR bands was apparent for the incubation times of 30–60 mins (Fig. 5). For comparison, with the same concentration of NaOH, 5 mins were enough for a complete degradation of viral RNA (Fig. 2). Therefore, these data showed clearly the difference in the detection of degradation of viral RNA by gel electrophoresis and RT-PCR.

Since the degradation of intact TMV virions was determined by the abovementioned methods, it was our interest to compare the observed degradation of virion or its RNA with the virus infectivity. Table 1 presents the results of the infectivity assay by local lesion production after treatment of the virus with HCl or NaOH. When TMV was exposed to 0.2N HCl, the number of local lesions in *N. rustica* was reduced within 15 mins by more than 95%.

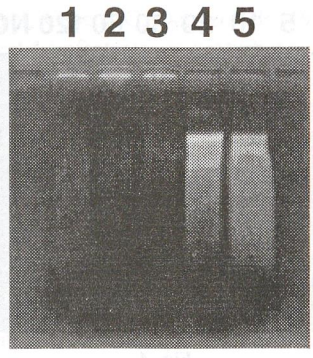

Fig. 1
Electrophoresis in 1% agarose gel of TMV (30 µg per lane) exposed to various concentrations of HCl for 30 mins
Non-treated TMV (lane 1) and TMV treated with 0.01 N (lane 2), 0.1 N (lane 3), 0.2 N (lane 4), and 0.3 N HCl (lane 5).

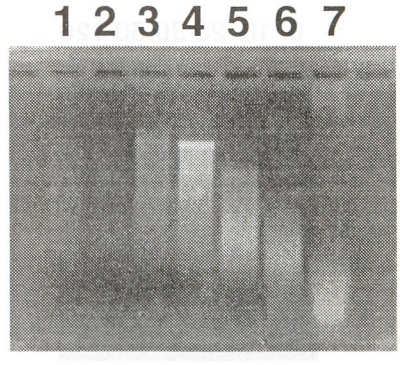

Fig. 2
Electrophoresis in 1% agarose gel of TMV exposed to various concentrations of NaOH for 5 mins
Non-treated TMV (lane 1) and TMV treated with 0.01 N (lane 2), 0.02 N (lane 3), 0.05 N (lane 4), 0.1 N (lane 5), 0.2 N (lane 6), and 0.5 N NaOH (lane 7).

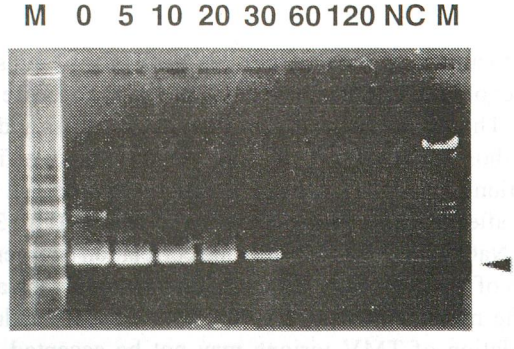

Fig. 3
Detection of RT-PCR products of TMV treated with 0.2 N HCl for various times
DNA ladder (1 kb, left M lane), 0 min (lane 0), 5 mins (lane 5), 10 mins (lane 10), 20 mins (lane 20), 30 mins (lane 30), 60 mins (lane 60), and 120 mins (lane 120). No TMV (lane NC). *Hind*III-digested lambda DNA (right M lane). The 806 bp product (arrowhead).

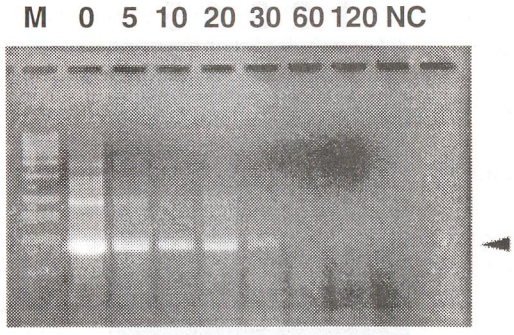

Fig. 4
Detection of RT-PCR products of TMV treated with 0.3 N HCl for various times
DNA ladder (1 kb, lane M), 0 min (lane 0), 5 mins (lane 5), 10 mins (lane 10), 20 mins (lane 20), 30 mins (lane 30), 60 mins (lane 60), and 120 mins (lane 120). No TMV (lane NC). The 806 bp product (arrowhead).

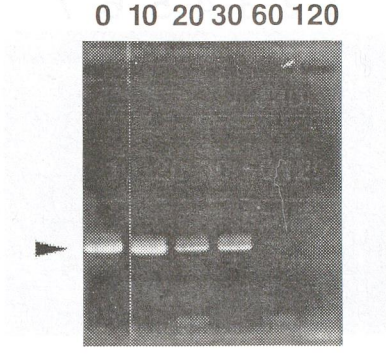

Fig. 5
Detection of RT-PCR products of TMV treated with 0.1 N NaOH for various times
0 min (lane 0), 10 mins (lane 10), 20 mins (lane 20), 30 mins (lane 30), 60 mins (lane 60), and 120 mins (lane 120). The 806 bp product (arrowhead).

After treatment of TMV with 0.1 N NaOH for 10 mins, the number of local lesions was reduced similarly (by more than 97%). Therefore, the results of the infectivity assay differ from those of the RT-PCR assay, in that no significant reduction in the RT-PCR band intensity was observed with TMV after 30 mins of exposure to 0.2 N HCl (Fig. 3) or 0.1 N NaOH (Fig. 5). Therefore, no correlation between the results of the two compared methods was found indicating that the measurement by RT-PCR of chemically-induced degradation of TMV virions may not be accepted as a criterion for TMV (infectivity) inactivation.

Other disinfectants were also shown to be efficient in reducing the TMV infectivity. The concentration of formalin and phenol recommended for use as a disinfectant is as low as 2%. We observed that a treatment with 2% formalin resulted in reduction of viral infectivity below the detection limits within 30 mins of exposure, and that a treatment with 2% phenol caused a 90% inhibition.

We also evaluated the degrading effect of UV irradiation on TMV capsid and RNA, because many laboratories and storage houses frequently use UV light to decontaminate infectious agents. As we expected, formalin, phenol, and UV light effectively reduced the TMV infectivity, but a corresponding degradation of TMV RNA was demonstrated by neither gel electrophoresis nor RT-PCR (data not shown).

Along with gel electrophoresis, the efficacy of RT-PCR was demonstrated to be effective in detecting degraded TMV particles. Nevertheless, even though PCR is recognized as a rapid and sensitive method for virus detection, our results further emphasize that an infectivity assay should be employed to avoid false-positive results which were observed by us in this study or reported by other authors (Ma *et al.*, 1994; Moore and Margolin, 1994).

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