Inhibitory Effect of Gamma Irradiation against *Cucumber Green Mottle Mosaic Virus*

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Abstract

Hong J.-S., Jeong M.-A., Jeong R.-D. (2017): Inhibitory effect of gamma irradiation against *Cucumber green mottle mosaic virus*. Plant Protect Sci., 53: 201–207.

Gamma irradiation has been shown to be an effective method of controlling plant bacterial and fungal pathogens, but data on its effect against plant viruses is limited. A mechanism for the inactivation of plant viruses by gamma irradiation has not been proposed. Gamma irradiation was evaluated for the inactivation of *Cucumber green mottle mosaic virus* (CGMMV) in *Nicotiana tabacum* plants. CGMMV infectivity decreased gradually in a dose-dependent manner, and the virus was completely inactivated at over 40 kGy. Transmission electron microscopy revealed that gamma irradiation disrupts the virion structure and degrades viral protein and genomic RNA, suggesting that damage to viral constituents is the mechanism by which gamma irradiation inactivates the virus.

Keywords: coat protein; ionising radiation; virus inactivation; virion

Plant viral diseases cause severe economic losses in agriculture. The *Cucumber green mottle mosaic virus* (CGMMV), a member of the genus *Tobamovirus*, is an economically significant viral pathogen infecting cucurbit species. It causes systemic mosaic and mottling on leaves, blistering, deterioration of fruit pulp, and dwarfism, resulting in major economic losses. CGMMV has a 6.4 kb (+)ssRNA genome and rod-shaped virions, approximately 300 nm in length and 18 nm in diameter (Varveric *et al.* 2006). Similar to other viruses, the CGMMV virion replicates itself by entering a host cell, disassembling itself and copying its components. CGMMV is the most persistent plant virus known, surviving up to 50 years in dried plant parts. Although chemical control is effective against many fungal and bacterial pathogens, to date no efficient chemical treatment has been developed for controlling the CGMMV infection. An alternative approach would be to develop transgenic plants containing constructs targeting the endogenous genes of CGMMV. However, transgenic technology has not yet been commercially accepted (Gloemboski *et al.* 1990). Therefore, it is urgent to develop a novel, effective, and eco-friendly treatment to inactivate viral pathogens.

One potential nonchemical technology for the inactivation of viral pathogens is irradiation technology. It has been suggested that ionising radiation might be used to disinfect bacteria, insects, fungi, and other pests without incurring significant risks to human health or the environment (Feng *et al.* 2011; Predmore *et al.* 2015). Three types of irradiation (i.e., gamma, electron beam, and X-ray irradiation) are now commercially employed for disinfecting pathogens with gamma and electron beam irradiation being the most commonly used.

Gamma irradiation, which is derived from a cobalt source, has much higher penetrability into materials than does electron beam irradiation, which is generated through the acceleration and conversion of electricity. The exposure of microorganisms to ionising radiation directly damages the cellular macromolecules (nucleotides, ribonucleotides, and proteins to a lesser extent), or indirectly attacks the target macromolecules through the generation of a substantial flux of free oxygen radicals (especially hydroxyl radicals and hydrogen peroxide) (Smolko & Lombardo 2005). The unit of irradiation dose is the gray (Gy), which is the energy absorbed in J/kg of the material.

Gamma irradiation has been used to inhibit the growth of some plant pathogenic fungi such as *Botrytis cinerea*, *Penicillium expansum*, *P. italicum*, *Rhizopus stolonifer* var. *stolonifera,* and *Monilinia fructicola* (Kim & Yook 2009; Chu *et al.* 2015; Jeong *et al.* 2015). However, the feasibility of using gamma irradiation to inactivate plant viruses in crops has not been established. To date, no study has investigated the inactivation of plant viruses by gamma irradiation. Moreover, the mechanism of viral inactivation by gamma irradiation is poorly understood.

In this study, we evaluated the effectiveness of gamma irradiation for the inactivation of CGMMV over a range of doses. We also monitored the infectivity of gamma-irradiated CGMMV in locally and systemically infected tobacco leaves. Furthermore, we investigated possible mechanisms of viral inactivation by gamma irradiation using transmission electron microscopy (TEM) of virus particles and analysis of viral protein and genomic RNA. Our goal was to evaluate the potential usefulness of gamma irradiation for the control of plant viruses.

MATERIAL AND METHODS

CGMMV preparation and inoculation*. Cucumber green mottle mosaic virus* (CGMMV-KW) was obtained from the Plant Virus GenBank (PVGB) of Korea. CGMMV was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) with specific primers of coat protein designed from previously reported CGMMV sequences in GenBank (accession codes AB015146 and D12505), and PCR products were sequenced (Kim *et al.* 2003). The virus was purified from wildtype-infected leaves of *Nicotiana tabacum* var. Samsun using a method described previously

(Asselin & Zaitlin 1978). Purified virus particles were resuspended at a concentration of 2 mg/ml in 0.1 M phosphate buffer (pH 7.0) containing 5 mM EDTA. Aliquots were irradiated with various doses of gamma radiation. For each radiation dose, single leaves of twenty 5-weeks-old tobacco plants that had been stripped to two leaves were mechanically inoculated with 100 μg of irradiated CGMMV. In addition, 20 plants were mock-inoculated with phosphate buffer only. Carborundum was used as an abrasive. Following inoculation, plants were rinsed with a gentle stream of tepid tap water. The experiments were carried out in two independent rounds, with ten plants from each group in each round. Inoculated plants were kept at 32°C under 16/8-h day/night conditions.

Analysis of viral titre and observation of infection symptoms*.* For an analysis of viral titre, inoculated leaves of 20 plants per treatment, regardless of whether the leaves showed symptoms, were collected at 5 days post inoculation (dpi). Viral RNA was extracted, and the virus concentration was analysed as previously described (Asurmendi *et al.* 2007). The viral titre was estimated by measuring the optical density at 260 nm (OD_{260}) using a spectrophotometer. For CGMMV, the extinction coefficient was assumed to be 3. The titre is expressed in micrograms of CGMMV per 10 mg of fresh weight.

Symptoms of viral infection, consisting of interveinal chlorosis in young leaves followed by leaf darkening and curling, were observed daily. For the disease incidence, symptoms were recorded at 30 dpi. The average percentage of plants with symptoms from three independent experiments was calculated and a five-grade rating scale based on the amount of leaf area affected by the disease was used to measure the severity of CGMMV symptoms: 0 – no symptoms; 1 – faint mosaic, < 25% of leaf area; 2 – mosaic malformation, 26–50% of leaf area; 3 – severe mosaic, 51–75% of leaf area; 4 – severe mosaic, > 75% of leaf area. The disease severity index (DSI) was calculated as follows:

$$DSI = \Sigma n^b/(N - 1)T$$

where: n – number of leaves in each grade; b – grade; N – number of grades used in the scale; T – total number of leaves scored

Source of gamma irradiation*.* A cobalt-60 gamma irradiator at the Korean Atomic Energy Research Institute, Jeongeup, Korea (150 TBq capacity; ACEL, MDS Nordion, Canada) was used for the irradiation.

All of the absorbed doses were calibrated using alanine dosimeters with a diameter of 5 mm and a Bruker EMS 104 EPR analyser (both Bruker Instruments, Rheinstetten, Germany) was used to determine the free-radical signal. The radiation absorbed dose was 10 kGy/hour.

Transmission electron microscopy (TEM) of virus particles. Irradiated samples of purified virus were analysed using TEM to see if there was any physical damage to the virus particle. Briefly, samples were fixed on a copper grid (Electron Microscopy Sciences, Hatfield, USA) and negatively stained with 1% ammonium molybdate (Sigma-Aldrich). Fixed samples were analysed using a transmission electron microscope (JEOL Ltd, Tokyo, Japan) at 200 kV at the National Academy of Agricultural Science in Korea. For each grid, at least three fields of approximately 200 μm^2 were viewed. Images were taken and analysed using a US1000 CCD camera and GATAN MICROGRAPH software (Gatan Inc., Warrendale, USA).

Detection of CGMMV. For reverse transcription-PCR (RT-PCR), viral genomic RNA was extracted from CGMMV suspensions (either treated or untreated with gamma irradiation) using the RNeasy MiniKit (Qiagen, Valencia, USA), according to the manufacturer's instructions. A total viral protein concentration of 1 µg/µl was used for RNA extraction. The RNA was treated with DNase and subjected to RT-PCR using a Superscript III RNase H reverse transcriptase kit (Invitrogen, USA). The cDNA was then used as templates for the amplification of CGMMV coat protein (CP) using the primers 5'-ATGTCT-TACAGTATCACTCC-3' and 5'-TCAAGTTGCAG-GACCAGAGGT-3'.

For the northern blot analysis of CGMMV-CP, total RNA was isolated from CGMMV-infected tobacco leaves using TRIzol reagent (Invitrogen, USA), following the manufacturer's instructions. Fifteen micrograms of total RNA were denatured, separated on a 1.5% agarose gel, transferred to a nylon membrane (Immobilon-Ny+ Transfer Membrane; Millipore Co., Billerica, USA) through capillary blotting and probed with a CGMMV-CP PCR-DIG labelled probe. The probe DNA fragment was synthesised from the total RNA isolated from CGMMV-infected leaves and labelled by PCR using DIG-2'-deoxyuridine 5'-triphosphate (DIG-dUTP) according to the supplier's instructions (PCR DIG probe synthesis kit; Roche Diagnostic GmbH, Boehringer Mannheim, Germany). Prehybridisation, probe hybridisation, and development were performed according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

For western blotting, two micrograms of highly purified gamma-irradiated or non-irradiated virus particles were separated by 12% SDS-PAGE gel and subjected to an immunoblot analysis using α-CP (Agdia, Elkhart, USA). Immunoblots were developed using alkaline phosphatase-based colour detection.

Statistical analyses. Data were subjected to ANOVA at $P < 0.05$ using SPSS 13.0 for Windows (SPSS Inc., USA). Duncan's multiple range test was used to compare differences between mean values.

RESULTS

To investigate the ability of gamma irradiation to control the *Cucumber green mottle mosaic virus* (CGMMV), we inoculated *N. tabacum* leaves with 200 ng of purified CGMMV particles that had previously been treated or not with gamma irradiation at a range of doses (10–50 kGy; 20 plants per dose). At 5 dpi, leaves inoculated with non-irradiated or irradiated (10–30 kGy) CGMMV particles exhibited disease symptoms (interveinal chlorosis). However, leaves inoculated with > 40 kGy did not show any symptoms. The number of lesions in the primary leaves decreased with increasing radiation dose (Table 1). The appearance of symptoms probably correlates with the presence of the virus in the local leaves. Thus, it was decided to measure the virus titre at 5 dpi. Consistent with the pattern of symptoms, the CGMMV concentration was significantly decreased in a dose-dependent manner ($P<0.05$), declining to

Table 1. Symptoms of CGMMV infection in primary and secondary leaves of *Nicotiana tabacum* 30 days after inoculation with CGMMV that had received gamma irradiation at a range of doses

Gamma irradiation (kGy)	Symptoms in	
	1st leaves	2nd leaves
0 (WT control)	+[a]	+
10	+	+
20	+	+
30	+/–	+/–
40	–	–
50	–	–

[a]or all doses; + more than 5 symptomatic lesions per cm^2; +/– less than 5 lesions per cm^2; – no lesions
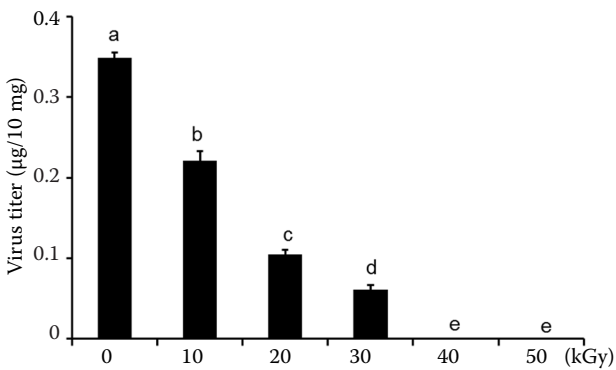
Figure 1. Plants exhibit a lower viral titre after inoculation with gamma irradiated CGMMV. Single leaves of 5-weeks-old *Nicotiana tabacum* plants (20 plants per treatment) were inoculated with 200 ng of gamma irradiated CGMMV. Virus concentration was measured in infected leaves at 5 dpi. The graph depicts average viral concentration from three independent experiments (in µg/ml CGMMV with SD). Bars sharing the same letter are not significantly different ($P < 0.05$) by Duncan's multiple range test ($n = 3$)

non-detectable levels at doses ≥ 40 kGy (Figure 1). A decline in the viral titre with increasing radiation dose has been observed in foodborne human viruses (Feng *et al.* 2011; Predmore *et al.* 2015). In order to further evaluate the susceptibility of *N. tabacum* plants to irradiated CGMMV particles using an *in vivo* assay, we measured the incidence of infection and severity of disease symptoms in systemic leaves at 30 dpi. Disease incidence gradually decreased with increasing radiation dose ($P < 0.05$) (Figures 2A and 2B). Symptom severity, evaluated on a standard scale of 0, also gradually decreased in systemic leaves in a dose-dependent manner ($P < 0.05$). In the 10-, 20-, and 30-kGy treatments, disease symptoms were mild to moderate. However, CGMMV samples treated with gamma irradiation of ≥ 40 kGy showed no lesions in the inoculated leaves and no CGMMV-CP transcript in the systemic leaves (Figures 2A and 2D). The experiment was repeated twice with the same outcome.
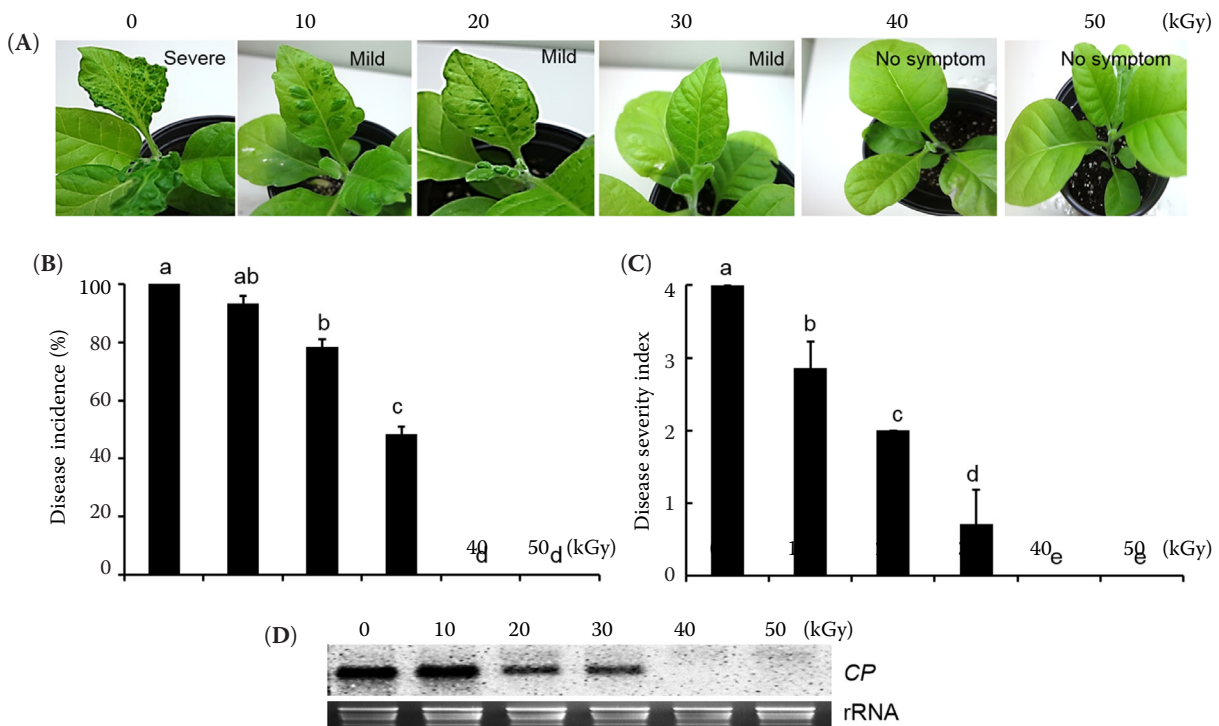

Figure 2. Inhibition of viral infection in *Nicotiana tabacum* plants inoculated with gamma irradiated CGMMV: (**A**) CGMMV symptoms. Plants were inoculated with CGMMV that had been treated with the indicated dose of gamma irradiation. 20 plants were inoculated in each of three independent experiments. Photographs were taken at 30 dpi; (**B**) CGMMV incidence and disease severity index (**C**) measured at 30 dpi. Disease incidence refers to the percentage of infected plants. See Methods for the explanation of disease severity index. Bars sharing the same letter are not significantly different ($P < 0.05$) by Duncan's multiple range test ($n = 3$); (**D**) systemic spread of gamma-irradiated CGMMV to the uninoculated tissue of CGMMV-infected plants. RNA was extracted from systemic tissue at 20 dpi and analysed for the presence of viral coat protein transcripts (*CP*)
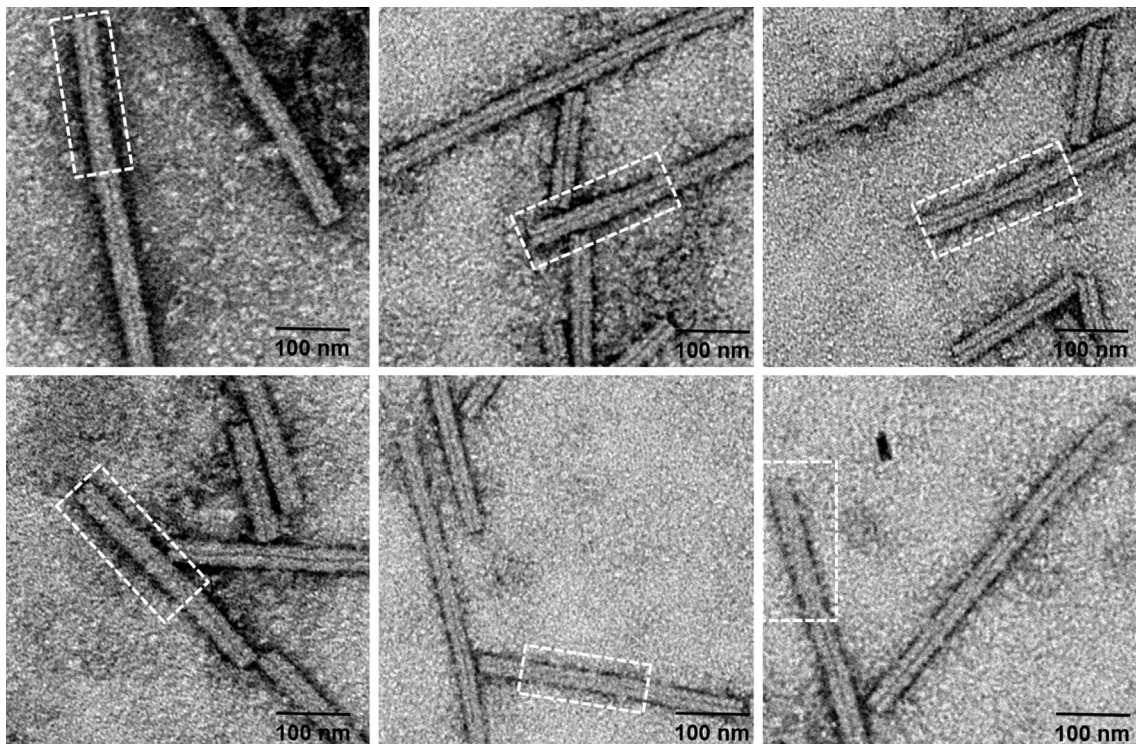
Figure 3. Gamma irradiation damage to CGMMV. Purified CGMMV was irradiated at doses of 10, 20, 30, 40, and 50 kGy. Treated and untreated virus particles were negatively stained with 1% ammonium molybdate and observed by transmission electron microscopy

To determine whether the gamma irradiation directly damages CGMMV particles, we analysed the virus particles using TEM. Considerable damage to the viral coat protein was shown in the irradiated virus particles compared to the non-irradiated particles. For the untreated control, CGMMV particles were rod-shaped virions of 300 nm in length and 18 nm in diameter, with no visible damage (Figure 3). Treatment with radiation ≥ 10 kGy significantly reduced the number of virus particles. Moreover, the quality of microscopy stain visible around the virus particles gradually decreased with increasing radiation dose (Figure 3), suggesting that the viral coat protein was being degraded by the gamma irradiation. Subsequently, the purified CGMMV was treated with the same dosage range of gamma irradiation and was subjected to SDS-PAGE analysis with CGMMV-CP antibody to determine the effect of irradiation on viral proteins. As shown in Figure 4A, the abundance of CGMMV-CP protein gradually decreased with increasing dose of irradiation. In the 30-kGy treatment, the abundance of CP was reduced by more than 50% compared to the untreated control (Figure 4A). In the 40- and 50-kGy treatments, CGMMV-CP protein was undetectable, suggesting that it was completely degraded (Figure 4A). The TEM and SDS-PAGE results suggest that the gamma irradiation treatment degrades viral proteins. In view of the CGMMV coat protein degradation by gamma irradiation, it was important to determine the effect of irradiation on viral genomic RNA. RNA extracted from purified CGMMV treated with the same dosage range of gamma irradiation was subjected to RT-PCR to amplify the *CGMMV-CP* gene, and the products were visualised through gel electrophoresis. As shown in Figure 4B, the *CP* gene was amplified in RNA samples extracted from CGMMV that had been treated with 30 kGy, although the abundance of the *CP* gene decreased with increasing dosage of radiation. No bands were detected for CGMMV treated with 40 or 50 kGy. Moreover, to confirm the finding of CGMMV inactivation, the integrity of the viral genome RNA in irradiated samples was investigated by electrophoresing. 20 μg of extracted RNA treated with gamma irradiation was loaded on 1% agarose gel. As shown in Figure 4C, CGMMV viral RNAs were gradually degraded at radiation doses of 30 kGy, and completely degraded at 40 and 50 kGy, suggesting that the *CP* gene was degraded by gamma irradiation.
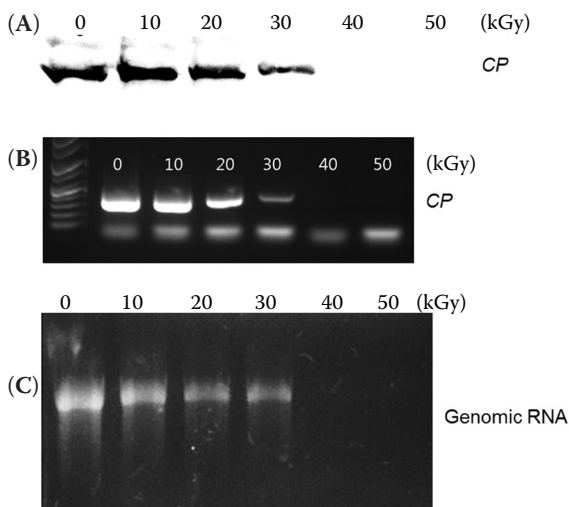
Figure 4. Analysis of CGMMV after gamma irradiation at doses of 0, 10, 20, 30, 40 or 50 kGy: (**A**) Western blot analysis of the CGMMV capsid protein. Total proteins were separated by SDS-PAGE and subjected to Western blotting using a CGMMV CP antibody; (**B**) RT-PCR detection of the CP gene in CGMMV genomic RNA; (**C**) Gamma irradiation degrades CGMMV genomic RNA. Total genomic RNA was isolated from purified CGMMV particles, and equal quantities of purified RNA were separated by electrophoresis

DISCUSSION

Irradiation has been found to be an effective processing technology for the control of fungi, bacteria, insects, and other pests, and it has been found to pose no significant risks to either human health or environment (Blank *et al.* 1995). However, the feasibility of using gamma irradiation to eliminate viruses in plants has not been established. In this study, we evaluated the effectiveness of gamma irradiation for the inactivation of CGMMV over a range of irradiation doses. In addition, we investigated possible mechanisms of viral inactivation by gamma irradiation. We found that CGMMV was significantly inactivated by gamma irradiation, and that the efficacy was positively correlated with the gamma irradiation intensity. Gamma irradiation degraded viral structural proteins and genetic materials and damaged viral particles of CGMMV, resulting in viral inactivation. Thus higher doses of gamma irradiation resulted in a greater amount of damage to the CGMMV virion and its genome. So far, only a few studies have investigated the effects of irradiation on plant viruses as a means of viral inactivation. Only UV treatment has been employed to inactivate certain plant viruses

including *Tomato bushy stunt virus* (TBSV), *Tobacco necrosis virus* (TNV), *Cowpea mosaic virus* (CPMV), and *Barley stripe mosaic virus* (BSMV) (Bawden & Kleczkowski 1953; Rae *et al.* 2008; Urban *et al.* 2011). When total UV doses of ≥ 100 mJ/cm^2 were applied, UV-irradiated BSMV did not cause any local lesions in *Chenopodium amaranticolor*, whereas non-irradiated BSMV produced > 200 local lesions. When the irradiated viral particles were examined by TEM, considerable damage to the viral coat protein was observed together with an increase in the amount of microscopy stain surrounding and penetrating into the virus particles (Urban *et al.* 2011). Another study found that short-wave (254 nm) UV irradiation inactivated CPMV infectivity, inhibited symptoms on inoculated leaves and degraded viral genomic RNA and particles (Rae *et al.* 2008). However, gamma irradiation has yet to be used for the commercial inactivation of plant viruses. This is partly due to the fact that the dose required to inactivate viruses is quite high (generally more than 5 kGy), owing to the size of the viral genome and the lack of water in the genome. Another reason is that a high dose (10–50 kGy) of gamma irradiation cannot be used to treat virus-infected crops directly because of resulting physiological damage to the crop. However, there is a possibility that lower doses of gamma irradiation can be combined with chemical treatment to inactivate viral pathogens, an approach that has been shown to be effective against postharvest fungal pathogens of cut roses and pears (Chu *et al.* 2015; Jeong *et al.* 2015).

Our finding that the virion and viral genome of CGMMV can be degraded by gamma irradiation is in agreement with reports on other viruses. The foodborne virus, *Tulane virus* (TV) and the human viruses, *Murine norovirus-1* (MNV-1) and vesicular stomatitis virus (VSV) exhibited decreased amounts of capsid protein after irradiation treatment (Feng *et al.* 2011; Predmore *et al.* 2015). The sensitivity of proteins to gamma irradiation is related to their amino acid content. Amino acids containing sulphur (e.g., cysteine, methionine) or aromatic compounds (tyrosine, phenylalanine) show high sensitivity because they react with hydroxyl radicals more easily than aliphatic (alanine, leucine, valine) amino acids (Stewart 2001). In addition, aggregation or crosslinking of proteins occurs during irradiation, resulting in the disruption of secondary and tertiary structures (Stewart 2001). It is also known that gamma irradiation can cause random breaks or le-

sions in the genetic material of microorganisms and that the formation of hydroxyl radicals and hydrogen peroxide during radiolysis of water can break bonds that bind nucleic acids together (Dickson 2001). Factors influencing viral sensitivity to gamma irradiation include genome size, type of enveloped virus, complexity of the virus structure, and the size of the virus particle (Feng *et al.* 2011).

Taken together, our results showed that gamma irradiation was effective at inactivating CGMMV, and that possible mechanisms of this inactivation include disruption of the virion structure and degradation of viral proteins, and genomic RNA. A better understanding of the mechanism of virus inactivation will facilitate the proper application of irradiation in industry.

References

Asselin A., Zaitlin M. (1978): Characterization of a second protein associated with virion of Tobacco mosaic virus. Virology, 91: 173–181.

Asurmendi S., Berg R.H., Smith T.J., Bendahmane M., Beachy R.N. (2007): Aggregation of TMV CP plays a role in CP functions and in coat-protein-mediated resistance. Virology, 366: 98–106.

Bawden F.C., Kleczkowski A. (1953): The behaviour of some plant viruses after exposure to ultraviolet radiation. Journal of General Microbiology, 8: 145–156.

Blank G., Corrigan D. (1995): Comparison of resistance of fungal spores to gamma and electron beam radiation. International Journal of Food Microbiology, 26: 269–277.

Chu E.H., Shin E.J., Park H.J., Jeong R.D. (2015): Effect of gamma irradiation and its convergent treatment for control of postharvest *Botrytis cinerea* of cut roses. Radiation Physics and Chemistry, 115: 22–29.

Dickson J.S. (2001): Radiation inactivation of microorganisms. In: Molins R. (ed.): Food Irradiation: Principles and Applications. New York, Wiley-Interscience: 23–35.

Feng K., Divers E., Ma Y., Li J. (2011): Inactivation of a human norovirus surrogate, human norovirus virus-like particles, and vesicular stomatitis virus by gamma irradiation. Applied and Environmental Microbiology, 77: 3507–3517.

Golemboski, D.B., Lomonossoff, G. P., Zaitlin, M. (1990): Plants transformed with a Tobacco mosaic virus nonstructural gene sequence are resistant to the virus. Proceeding of the National Academy of Sciences of the United States of America, 16: 6311–6315.

Jeong R.D., Chu E.H., Shin E.J., Lee E.S., Kwak Y.S., Park H.J. (2015): Antifungal effect of gamma irradiation and sodium dichloroisocyanurate against *Penicillium expansum* on pears. Letters in Applied Microbiology, 61: 437–445.

Kim K.H., Yook H.S. (2009): Effect of gamma irradiation on quality of kiwifruit (*Actinidia deliciosa* var. *deliciosa* cv. Hayward). Radiation Physics and Chemistry, 78: 414–421.

Kim S.M., Lee J.M., Yin K.O., Oh M.H., Park J.W., Kim K.H. (2003): Nucleotide sequences of two Korean isolates of *Cucumber green mottle mosaic viru*s. Molecules and Cells, 16: 407–412.

Predmore A., Sanglay G.C., DiCaprio E., Li J., Uribe R.M., Lee K. (2015): Electron beam inactivation of *Tulane virus* on fresh produce, and mechanism of inactivation of human norovirus surrogates by electron beam irradiation. International Journal of Food Microbiology, 198: 28–36.

Rae C., Koudelka K.J., Destito G., Estrada M.N., Gonzalez M.J., Manchester M. (2008): Chemical addressability of ultraviolet-inactivated viral nanoparticle (VNPs). PLoS ONE, 3(10): e3315. doi: 10.1371/journal.pone.0003315

Smolko E.E., Lombardo J.H. (2005): Virus inactivation studies using ion beams, electron and gamma irradiation. Nuclear Instruments and Methods in Physics Research Section B, 236: 249–253.

Stewart E.M. (2001): Food irradiation chemistry. In: Molins R. (ed.): Food Irradiation: Principles and Applications. New York, Wiley-Interscience: 37–76.

Urban M., Motteram J., Jing H.C., Powers S., Townsend J., Devonshire J., Pearman I., Kanyuka K., Franklin J., Hammond-Kosack K.E. (2011): Inactivation of plant infecting fungal and viral pathogens to achieve biological containment in drainage water using UV treatment. Journal of Applied Microbiology, 110: 675–687.

Varveric C., Vassilakos N., Bem F. (2006): Characterization and detection of *Cucumber green mottle mosaic virus* in Greece. Phytoparasitica, 30: 493–501.