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The antiviral effect of proteins and peptides derived from potatoes

朝鮮大學校　大學院

生物新素材　學科

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朴　載　均

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

BSA	Bovine Serum albumin
CP	Coat (Capsid) protein
DAS	Double-antibody sandwich
DPI	Days post inoculation
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Extreme resistance
HC-pro	Helper component proteinase
HR	Hypersensitive resistance
LRR	Leucine-rich repeat
NBS	Nucleotide binding domain
NIa	Proteinase part of NIa
OD	Optical density
PBST	Phosphate buffered saline-0.05% Tween 20
PLRV	Potato Leafroll Virus
PVY	Potato virus Y
PVY^{O}	Potato virus Y (ordinary strain)
Q-PCR	Quantitative-PCR or Real-time PCR
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
Ry	Resistant to potato virus Y
SAR	Systemic acquired resistance
VPg	Viral genome-linked Protein

ABSTRACT

The antiviral effect of proteins and peptides derived from potatoes

(감자로부터 유도된 단백질과 펩타이드의 항바이러스성 효과)

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Molecular genetic research has been identifying the genes that could considerably increase crop yields through transgenic-mediated resistant to viral, insect, fungal and bacterial pathogens. Resistant to potato virus Y (*Ry*) gene confers extreme resistance to all strains of Potato Virus Y (PVY). To identify the virus resistant potato, 12 verities of Korean potato cultivars were infected by PVY^{O} with the help of syringe. Of which 10 varieties were showed similar results in *in vitro* and *in vivo* experiments. Virus infected potatoes showed symptoms like mosaic, yellowing, necrosis, mottle, vein clearing, vein bending etc. *Golden valley* potato was found resistance to PVY^{O} infection. Enzyme-Linked Immunosorbent Assay (ELISA) result also showed that *Golden valley* and *Winter valley* were found resistant and highly susceptible to the PVY^{O} virus respectively. RT-PCR and Quantitative-PCR result showed that *Ry* gene was overexpressed 2.75 times more in PVY^{O}

infected *Golden valley* than the non-infected ones. PVY^{O} virus movement was also observed in *Winter valley* potato after the 7, 14 and 21 dpi. Virus was moved rapidly toward growing regions of the plant parts. Potato protein and peptides from virus resistant potato *Golden valley* and *Gogu valley* showed antiviral activity. Alternatively, the development of biotic pesticide work is going on.

I. INTRODUCTION

The potato (*Solanum tuberosum* L.*)* is widely grown edible crop ranking on fourth food after the wheat, rice and maize. Potato virus is a hazardous disease to potato, which may decrease the yields from 15 to 75 percent and make misshapen tubers or internal discoloration (Fang *et al*., 2005; Hull, 1984; Debokx and Huttinga, 1981). *Potato Virus Y* (PVY), the type member of the genus *Potyvirus* in the family *Potyviridae* (Shukla *et al*., 1994) is a widespread virus leading to severe damage in solanaceous crops like potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), green pepper (*Capsicum annuum*), tobacco (*Nicotiana tabacum*) etc.

Virus can be described as basically nucleic acid, DNA or RNA, surrounded by a coat or capsid protein (CP), which can not be visualized without use of an electron microscope. Viruses reproduce themselves inside the host cell. Viruses can be spread by different vectors like fungi, insects, nematodes, leaf hoppers, and mechanical means.

PVY shows flexuous, not-enveloped, rod-shaped particles 740 nm length and 11 nm width. PVY genome consists of single-stranded positive sense RNA of approximately 10.4 kb in genome size, which translates to form a single poly protein from which eight proteins are cleaved (Agrios, 2005; Boonham *et al*., 1998; Debokx and Huttinga, 1981)**.** The RNA genome of PVY has a VPg (viral genome-linked protein) covalently bound to its 5' terminus, and a poly (A) tail at its 3' terminus, but not essential for infectivity.

Potato viruses in leaf sap are readily detected by ELISA, RT-PCR and Quantitative (Real-time) PCR.

Infection of PVY is generally mediated by aphid transmission. It has been known that there are following three major strains of PVY (Jones, 1990). (i) PVY^{O} (ordinary or common strain) is widely distributed throughout the potato growing areas of the world. This strain induces mild to sever mosaic, mottling or yellowing, leaf drop and premature death and can cause stem necrosis in some cultivars. Necrosis can appear as spots or rings on leaflets. Plants grown from infected tubers (secondary infection) may show the same symptoms as well as stunting, but the symptoms tend to be less sever than these on plants infected with early current-season inoculum. Infected tubers have no symptoms in most cases. Occasionally light brown necrotic rings are visible on the skin. (ii) The PVY^{C} is designated as the stipple streak strain and produces systemic mosaic in some cultivars and induces hypersensitive reaction in other cultivars. PVY^{C} strains including potato virus C, are probably present in Australia, India and in some parts of the U.K. and continental Europe. (iii) PVY^{N}, the 'N' strain of PVY produces milder forms of mottling but it is still capable of reducing yield by up to 30 %. PVY^{N} strains occur in Europe including the former U.S.S.R., parts of Africa and South America.

Two main types of PVY-resistant potato are extreme resistance (ER) and hypersensitive resistance (HR) (Sabina *et al*., 2002). ER protects potato plants against all strains of PVY. It strongly suppresses virus accumulation in infected cells, and no visible symptoms or detectable amounts of PVY are observed in inoculated plants (Pere *et al*., 2003; Hamalainen *et al*., 1998;

Barker and Harrison, 1984; Cockerham, 1970; Ross, 1952). In contrast, HR to PVY is characterized by development of necrotic lesions (cell death) at the initial infection sites on inoculated leaves. For example, *Ny* confers HR to the PVY^{O} (Jones, 1990; Cockerham, 1970). HR may prevent the spread of virus within and from the inoculated leaf, but the resistance mechanism itself is unknown yet. In some cases, HR may be activated but fails to restrict virus movement in plant tissues, which results in the development of larger necrotic lesions, vein necrosis, or lethal necrosis in the inoculated leaf. Higher temperatures can modulate expression of HR, reduce its efficiency to restrict viral movement, and cause extensive tissue necrotization symptoms (Valkonen *et al*., 1998; Valkonen, 1997; Adams *et al*., 1986; Dykstra, 1939).

PVY is the most important cause of disease in potato and it cause significant loss in yield. Therefore development of the virus resistant potato species is felt necessary.

II. MATERIALS AND METHODS

II - 1. Plant materials and *in vitro* stem propagation

The virus-free 12 different cultivars of Korean valley potato were kindly provided by company of Potato valley. These tubers were propagated first on sterilized soil (Mix 5 Soil, Sun Grow Horticulture, Canada). Plantlets were obtained within 2 to 4 weeks. Healthy plantlets were selected for *in vitro* propagation. Plantlets were soaked in 70 % Ethanol for 2 minutes, followed by surface sterilization using 3 % clorax for 10 minutes. Plantlets were washed with 3rd distilled water (DW) for 3~5 times and excised into segments (1-1.5 cm) which contain the node with axillary buds. Moisture of explants were soaked in sterilized blotting paper and then cultured in MS medium (3 % sucrose, 0.8 % agar and pH 5.8) (Murashige and Skoog, 1962). Every 4 weeks, the plants were propagated in new MS medium. Potato plants were grown in a growth room at 22℃ with a light dark-cycle of 16/8 h.

II - 2. Virus infection

PVY^{O} ampoule was kindly provided by Plant Virus Genbank, Seoul Women's University, Korea. The ampoule was ground in autoclaved dry mortar and pestle with 1:100 (W/V) 0.1M Sodium-Phosphate buffer (pH 7.0). PVY^{O} expression was achieved by injection of Potato leaves with Syringe (Korea vaccine co., LTD, 26GX1/2) (Fig. 1). After 7 days post inoculation (dpi) virus infections were tested in third upper and lower from the primary infected ones.

II - 3. Total protein assay

Total proteins were assayed in a 50 mM Tris-HCl buffer (pH 7.5) using the Bradford's method. Bovine serum albumin (BSA, New England BioLabs, Inc) was used to generate a standard curve. All measurements were carried in microplate in triplicates. The absorbance was measured at 595 nm by using a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA) as described by Park *et al*. (2004). We have used 10 µg/ 100 µl proteins for ELISA test.

II - 4. ELISA test for detect PVY^{O} infected potato varieties

The virus status of the *in vitro* plantlets was determined by DAS-ELISA, as described by Clark and Adams (1977). DAS-ELISA (Agdia Inc., Elkhart, IN,

USA) was used for detection of PVY^{O}. All the reagents were obtained commercially (Agdia, Inc, USA) and were used at 100 µl per well in microtitre plates (Nunclon delta surface, Denmark). Potato leaf disk was put in disposable sample extraction bag (Agdia, Inc, ACC 00930) in ten-fold general

A B

Fig. 1. PVY^{O} infection to *Winter Valley* leaf. PVY^{O} (5 µl from concentration of 10 µg/ µl), expression was achieved by insertion on to *Solanum tuberosum* L. leaves with syringe (KOREA vaccine co., LTD, 26GX1/2) from ventral surface of the leaves. A: Before insertion with PVY^{o}, B: After insertion with PVY^{o}.

extraction buffer (Agdia, Inc, ACC 00955) {1.3 g Sodium sulfite (anhydrous), 20.0 g Polyvinylpyrrolidone (PVP) MW 24-40,000, 0.2 g Sodium azide, 2.0 g Powdered egg (chicken) albumin, Grade II, 20.0 g Tween-20 per 1 liter, pH 7.4}. After grinding at room temperature, the lysate was centrifuged at 13,000 rpm for 15 minutes at 4 ℃. The supernatant was used as antigen. 100 µl Capture (coating) antibody (Agdia, Inc, CAB 20000, anti-rabbit) in 1X Carbonate coating buffer (Agdia, Inc, ACC 00413) {1.59g Sodium carbonate (anhydrous), 2.93 g Sodium bicarbonate, 0.2 g Sodium azide per 1 liter, pH 9.6} was applied to each well of the microtitre plate. The plate was then incubated at 4 ℃ overnight in a humid box. Washing the plate 2 times with 1X PBST buffer (Agdia, Inc, ACC 00115) (8.0 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, 0.5 g Tween-20 per 1 liter, pH 7.4), 100 µl of antigen was applied to each well and the plate was then incubated at room temperature for 2 hours. Plates were washed 4-8 times with 1X PBST buffer. After washing, upside frame was hold down and tapped firmly on a folded paper towel to remove all droplets of buffer. The solution from each well was discarded and add 100 µl of Perox enzyme conjugated antibody (Agdia, Inc, ECP 20000, anti-rabbit) diluted 1:100, mix 5X MRS component (Agdia, Inc, ACC 00522) with 1X PBST in a 1 : 4 ratio. After incubation for 2 hours at room temperature, each well of the plates was washed with PBST buffer and filled with 100 µl of 1X TMB peroxidase substrate solution (Agdia, Inc, ACC 00412) was then incubated the plate in a humid box for 20 minutes. The test wells were measured at an absorbance of 650 nm (OD_{650}) on a plate reader (Molecular Devices, USA). Wells in which a blue color developed indicates

positive result while having no significant color development indicate negative result. All assays were performed in triplicates.

II - 5. RT-PCR for detect PVY^{O} infected potato varieties

II – 5 - 1. Total RNA extraction

Total RNA was extracted from infected leaf tissue using TRIzol reagent according to the procedures of Life Technologies. Leaf tissue was ground in liquid nitrogen with an autoclaved dry mortar and pestle. A sample of the powdered tissue (approximately 100 mg) was extracted for total RNA. Total RNA was eluted in 20 μl of RNase-free water. The concentration and purity of the total RNA were calculated with absorbance at 260 and 280 nm using a spectrophotometer (Bio-Red, Smart Spec TM 3000) and gel electrophoresis on 1.5 % agarose gel.

II – 5 - 2. cDNA synthesis

cDNA was synthesized by using the Superscript II reverse-transcriptase (Life Technologies). 2~5 μg of total RNA and 1μl of the oligo dT (500 μg/ml) were mixed in the reaction tube and it was heated at 65 ℃ for 10 min for inactivation and quick chilled on ice. The 5X first strand buffer and 0.1 M

DTT were added and mixed contents of the tube were gently incubated at 42℃ for 2 min. 1 μl (200 units) of Superscript II was put into the tube and incubated at 42 ℃ for 50 min and inactivated by heating to 70℃ for 10 min. Synthesized cDNA was stored at –20 ℃.

II – 5 - 3. RT-PCR amplification

RT-PCR amplification was carried out on cDNA synthesized from infected potato leaves. For the *CP* gene amplification, the forward primer located in the coat protein gene position 86808708 according to the NCBI accession number NC_001616 and a reverse primer located downstream at position 93369365 were used. In order to *Ry* gene amplification, the forward and reverse primers according to the NCBI accession number AJ300266 were used (Table 1). All the primers were selected by computer analysis with the PRIME program. A 20 μl RT-PCR reaction mixture containing 2.5 mM dNTPs, 10 pmol of both primers, 1X Taq buffer, 1 μl cDNA and 1 unit Taq DNA polymerase was used for RT-PCR. *CP* gene was PCR amplified with a denaturation of 5 min at 94 ℃; 30 cycles at 94 ℃, 30 s; 57 ℃, 30 s, 72 ℃, 1.5 min; and a final extension of 5 min at 72℃. Similarly, for *Ry* gene was PCR amplified with a denaturation of 3 min at 94℃; 30 cycles at 94℃, 30 s; 55℃, 30 s; 72℃, 1 min.

Table 1. The oligonucleotide primers used for detection of virus infected potato varieties in RT-PCR.

Gene name	Primer A (Forward)	Primer B (Reverse)	Fragment (bp)
***CP* gene** (RT-PCR)	**YCPF :** 5'-AGGCACATCTGGGACA CATACTGTGCCGA-3'	**YCPR :** 5'-TGACTCCAAGTAGAG TATGCATACTTGGA-3'	690
***Ry* gene** (RT-PCR)	**Ry-1F :** 5'-GATGGCATCATCATCTT CTTCTTCTGA-3'	**Ry-1R :** 5'-CTTAGAGCTGTGACC TTTGTTTCTTAGA-3'	480
ß-actin	**ActinF :** 5'- GGCGATGAAGCTCAAT CCAAACG-3'	**ActinR :** 5'-GGTCACGACCAGCAA GATCAAGACG -3'	495

and a final extension of 5 min at 72 ℃. 20ul of amplified products were electrophoresed at 140 V for 20 min in a 1% agarose gel containing 0.5 mg·L^{-1} ethidium bromide (*EtBr*) and observed under ultraviolet light.

II - 6. Protein extraction from potato leaves

Total soluble protein was extracted from the potato leaves. Leaf materials were homogenized in liquid nitrogen and ground to powder, followed by adding 100 µl of protein extraction buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 150 mM NaCl, 0.1 % Triton X-100) to 100 mg of potato leaves. After grinding, the homogenate was centrifuged at 13,000 rpm for 15 minutes at 4 ℃ (Park *et al*., 2002). The supernatant was pooled and freeze-dried and obtained protein pellet. The protein pellet was dissolved in 3rd DW and assayed for further antiviral activity analysis.

II - 7. Peptide extraction from potato tubers

II – 7 - 1. Purification and characterization of G4 and G11 peptides

Potato tubers (*Solanum tuberosum* L cv. *Golden valley*) were stored at 4 ℃ in the dark at a relative humidity of 95 % to 100 % for up to 6 months. Potato tubers were soaked in distilled water for a few hours and then ground to a

fine powder in a coffee grinder. The resulting flour was suspended in extraction buffer containing 100 mM Tris-HCl and 1.5 M LiCl, pH 7.2. The supernatants were then dialyzed against distilled water, using membranes with a 1000 molecular weight cut-off (MWCO). The extract was heated at 70 ℃ for 20 min to obtain heat-stable peptides. Heat-denatured precipitates were removed by centrifugation for 30 min at 32,000 ×*g*. The supernatant was subjected to ultrafiltration with a 10,000 MWCO membrane, and the extract was purified by High Performance Liquid Chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) using a C_{18} reversed-phase column (4.6 × 250 mm; Vydac, Hesperia, CA, USA). Peptides were dissolved in 0.1 % (v/v) trifluoroacetic acid (TFA) in HPLC grade water (Solvent A) and loaded onto the C_{18} reversed-phase column that had been equilibrated with 0.1 % TFA. Peptides were eluted using a gradient of solvent B (acetonitrile with 0.1% TFA) in solvent A as follows: 0 to 25 min, 15 % solvent B; 25 to 85 min, 30 % solvent B; 85 to 90 min, 30 % solvent B; 90 to 110 min, 40 % solvent B; and 110 to 120 min, 40 % solvent B. The effluent from the column was monitored by measuring the absorbance at 214 nm. Each fraction was pooled and freeze-dried. To ensure that the collected peaks contained a single molecule, they were subjected to another round of purification. Each peak fraction was collected and assayed for antiviral activity. Peptide was kindly provided by the Research Center for Proteineous Materials (RCPM), Chosun University, Korea.

II – 7 - 2. Analogue 3 (A3) peptide synthesis

HP (2-20) peptide is the antimicrobial sequence derived from the N-terminus of *Helicobacter pylori* ribosomal protein L1 (RPL1). A3 peptide is analogue of HP (2-20) peptide which substituted by Trp for the hydrophobic amino acids Gln and Asp at positions 17 and 19 of HP (2-20) (Table 2). A3 peptides were synthesized by the solid phase method using Fmoc (9-fluorenyl-methoxycarbonyl)-chemistry. Rink amide 4-methyl benzhydrylamine (MBHA) resin (0.55 mmol/g) was used as the support to obtain a C-terminal amidate peptide. The coupling of Fmoc-L-amino acids was performed with N-hydroxybenzotriazole (HOBt) and dicyclohexycarbodiimide (DCC). Amino acid side chains were protected as follows: *tert*-butyl (Asp), trityl (Gln), *tert*-buty-loxycarbonyl (Lys). Deprotection and cleavage from the resin were carried out using a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1,2-ethandithiol and triisopropylsilane (88:2.5:2.5:2.5:2.5:2.0, v/v) for 2 h at room temperature. The crude peptide was then repeatedly washed with diethylether, dried in vacuum, and purified using a reversed phase preparative HPLC on a Waters 15 um Deltapak C_{18} column (19 x 30 cm). Purity of the peptide was checked by analytical reversed-phase HPLC on an Ultrasphere C_{18} column (Beckman, USA), 4.6 x 25 cm. The purified peptides were hydrolyzed with 6 N HCl at 110 ℃ for 22 h, and then dried in a vacuum. The residues were dissolved in 0.02 N HCl and subjected to amino acid analyzer (Hitachi Model, 8500A, Japan). Peptide concentration was determined by amino acid analysis. The molecular masses of the synthetic peptides were determined using the matrix-assisted laser desorption ionization (MALDI) mass spectrometer (Lee

et al. 2002). The peptide was also provided by the Research Center for Proteineous Materials (RCPM), Chosun University, Korea.

Table 2. Amino acid sequences of synthetic antimicrobial peptide.

Peptides	Amino acid sequences	Remarks
HP (2-20)	AKKVFKRLEKLFSKI**Q**N**D**K	native
Anal 3 (A3)	AKKVFKRLEKLFSKI**W**N**W**K	(Q^{17} D^{19} → W^{17} W^{19})

II - 8. *In vivo* treatment of proteins (*Golden valley* and *Gogu valley*) and peptides (G4, G11 and A3) to infected *Winter valley* leaves

3 weeks old *Winter valley* plants grown in soil were used. For treatment of *Golden valley* and *Gogu valley* proteins, *Golden valley* peptides and A3 peptide with PVY^O to virus susceptible *Winter valley* leaves, 500 µg amount of *Golden valley* protein was treated to *Winter valley*, after 10 minutes, virus was infected to *Winter valley* leaves. For treatment of peptide A3 with PVY^O to virus susceptible *Winter valley* leaves, same methods were used as above. 10 µg of A3 were applied to each *Winter valley* plant. 0.05 mg of virus from PVY^O ampoule was diluted 1:100 with 0.1 M sodium phosphate buffer (pH 7.0) and inoculated to each plant. PVY^O infection was achieved by infiltration of potato leaves with syringe. Inoculum was applied with a cotton swab on the upper side of two leaves per plants. Inoculated leaves were marked by piercing two holes in them. *Winter valley* plants without inoculation were used as negative controls.

II - 9. Quantitative (Real-time) PCR

Quantitative-PCR amplifications were carried out on cDNA from infected potato leaves. For the *CP* gene amplification, the forward primer located in

the coat protein gene position 86808708 according to the NCBI accession number NC_001616 and a reverse primer located downstream at position 93369365 were used. In order to *Ry* gene amplification the forward primer and reverse primer from the NCBI accession number AJ300266 was used (Table 3). All the primers were selected by computer analysis with the PRIME program. We used QuantiTect SYBR Green PCR Kit (QIAGEN) containing 2x SYBR Green PCR Master Mix with ROX as a passive reference dye, HotStarTaq DNA Polymerase and dNTP Mix. 5 pmol of both primers (Forward and Reverse), 1 µl cDNA and RNase-free water were added. In order to *CP* gene PCR amplified with a denaturation of 15 min at 95 ℃; 40 cycles at 95℃, 10 s; 54 ℃, 20 s, 72 ℃, 30 s; and a final extension of 5 min at 72℃. Similarly, for *Ry* gene PCR amplified with a denaturation of 15min at 95℃; 40 cycles at 95℃, 10 s; 52 ℃, 20 s; 72 ℃, 30 s; and a final extension of 5 min at 72 ℃. *CP* and *Ry* genes expression was measured on a real-time DNA detection system (Corbett co, RG-3000, Australia).

Table 3. The oligonucleotide primers used for detection of virus infected potato varieties in Quantitative-PCR.

Gene	Primer A (Forward)	Primer B (Reverse)	Fragment (bp)
CP gene *(*Quantitative -PCR)	**YCPRF :** 5'-TTGGTGCATTGAAAA TGGAA -3'	**YCPRR :** 5'- TTGCCTAAGGGTTGGT TTTG -3'	185
***Ry* gene** (Quantitative -PCR)	**Ry-1RF :** 5'-AGGCACCTGATGGCT CGTAG-3'	**Ry-1RR :** 5'-TGAAATGCTTGTCTCTC GTTGTTG-3'	154
ß-actin	**ActinF :** 5'-GGCGATGAAGCTCAA TCCAAACG-3'	**ActinR :** 5'-GGTCACGACCAGCAAG ATCAAGACG -3'	495

III. RESULTS

III - 1. Symtoms between potato varieties by PVY^O infection

We have used 12 potato varieties which showed similarities or different in their morphology. Those varieties can be differentiated with the tuber properties or leaves structures or height (Fig. 2).

III - 2. Screening of PVY^O resistant potato from 12 potato varieties by ELISA

Engvall and Perlman (1971) reported that ELISA is a highly versatile and sensitive technique, which can be used for qualitative or quantitative determinations of any antigen or antibody practically. If the optical density (OD) value of the virus-infected plants is 3 times higher than negative control, we determined that those plants are infected by virus. After 3 weeks of PVY^O infection, leaves from the main branch were tested by ELISA. As described in methods we examined the leaves up to 3 leaves below and up the leaf of infection. The OD value at 650 nm was different from each other (Fig. 3). Of the 12 potato varieties, the lowest OD value was from *Golden valley* and highest OD value from the *Winter valley*. The OD value of *Golden valley* was nearly equal to negative control, so we suggest that *Golden valley* is virus

resistant potato variety.

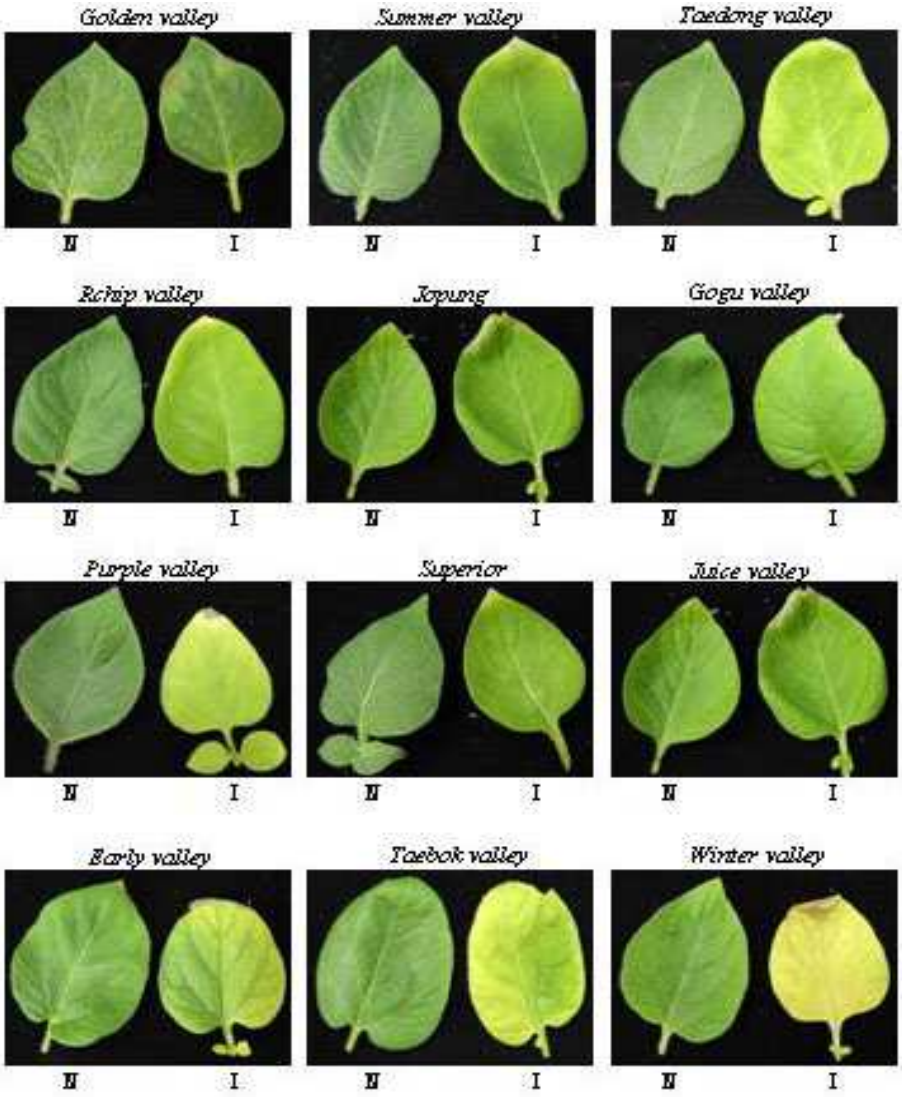

Fig. 2. The symptom of PVY^{O} non-infected (N) and infected (I) to 12 varieties of potato leaves. All the PVY^{O} infected leaves (right of each variety) are those above the inoculated leaf, taken from successive positions towards the tip from 21 dpi PVY^{O} infection. The non-infected leaf (left of each variety) is also same aged as PVY^{O} infected plant but they are taken from non-infected plants. Several local lesions

developed on the PVY^O infected *Taebok valley* and *Winter valley*.

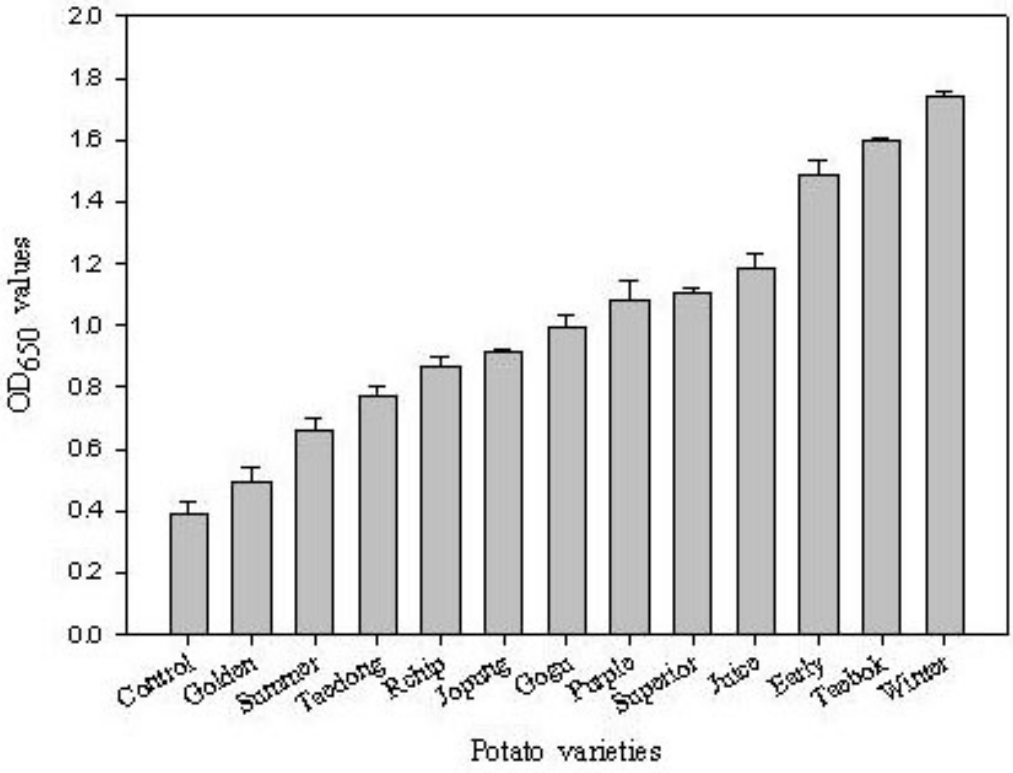

Fig. 3. DAS-ELISA spread of PVY^O in different cultivars. After the 21 dpi of PVY^O, OD_{650} value of each variety was measured as the average of five samples. Standard deviation indicated by error bars and are significantly different at $P<0.05$. The sap of mock inoculated *Winter valley* leaves was used as control.

III - 3. RT-PCR for virus detection in potatoes and

expression of *CP* and *Ry* genes

III – 3 - 1. Expression of CP gene

Coat or capsid protein (*CP*) gene is used in the assembly of virus particles and controls virus transmission by aphids. Recently CP gene was also found to be involved in virus spread by cell-to-cell movement. It is possible that the *CP* gene is able to increase the SEL (size exclusion limit) of plasmodesmata and to facilitate cell-to-cell movement of viral RNA. In order to detect expression of *CP* gene in PVY^O infected plants, RT-PCR was carried out with *CP* gene from 21 dpi of PVY^O to potato plants. As expected, *CP* gene was expressed in PVY^O infected *Winter valley* but not expressed in PVY^O infected *Golden valley* (Fig. 4). This result showed that *Winter valley* is susceptible to PVY^O and *Golden valley* is resistant to PVY^O.

III – 3 - 2. Expression of Ry gene

Resistant to PVY (*Ry*) gene confers extreme resistance (ER) to all strains of PVY. *Ry* gene is protects the plant against virus independently of cell death at the site of initial infection. To compare expression of *Ry* gene in virus-susceptible potato and virus-resistant potato varieties, RT-PCR was carried out with *Ry* gene from 21 dpi of PVY^O to potato plants. When *Winter valley*

was highly susceptible by PVY^O infection, *Ry* gene was less expressed (Fig. 4). When *Golden valley* leaves were infected by PVY^O, *Ry* gene was highly expressed than virus-free *Golden valley* plant (Fig. 5).

III - 4. Virus movement

To examine the extent to which direction PVY^O had moved faster, PVY^O was inoculated to the *Winter valley* leaf in middle part of the plant. After the 3 dpi of PVY^O movement was examined by measuring OD value in DAS-ELISA. Virus movement was detected at the interval of 3, 7, 14 and 21 dpi. After the 3 dpi virus expression was found in similar way in both apical and basal portion form the infected ones. After the 7 dpi to 21 dpi the virus movement was higher in basal than the apical leaves (Fig. 6).

III - 5. The effect of *in vivo* proteins and peptides treatment to PVY^O.

III - 5 - 1. *Golden valley* and *Gogu valley* proteins

To determine antiviral activity of proteins, 500 µg of each protein was applied to PVY^O infected *Winter valley* leaves. OD value was measured by ELISA reader. PVY^O inoculated *Winter valley* was used as control (Fig. 7).

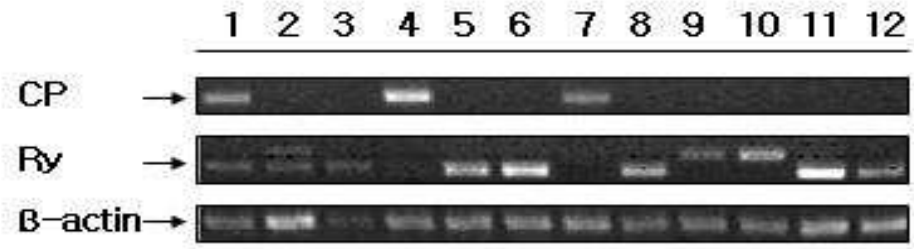

Fig. 4. Expression of *CP* and *Ry* genes in virus infected potato cultivar leaves by RT-PCR. After 21 dpi PVY^{O} infection, RT-PCR was carried out to for detection of *CP* and *Ry* gene. *CP* gene was detected in *Early valley*, *Winter valley* and *Taebok valley*. *Ry* gene was strongly detected in *Golden valley* and *Summer valley*. lane 1, *Early valley* ; lane 2, *Gogu valley;* lane 3, *Purple valley*; lane 4, *Winter valley* ; Lane 5, *Taedong valley*; lane 6, *Summer valley*; lane 7, *Taebok valley*; laen 8, *Jopung* ; lane 9, *Juice valley*; lane10, *Rchip valley*; lane 11, *Golden valley*; lane 12, *Superior*

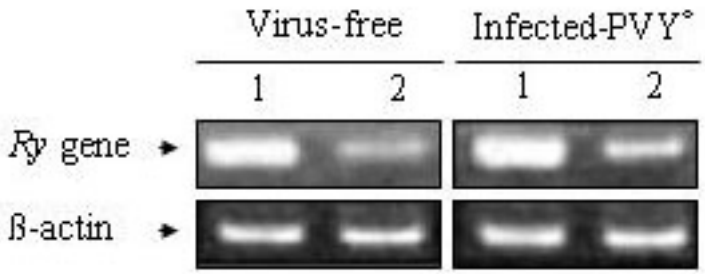

Fig. 5. Expression of *Ry* gene in PVY^{O} uninfected and infected potato leaves by RT-PCR. *Golden valley* show strong resistance to PVY^{O} before infection of PVY^{O}. However *Winter valley* was highly susceptible by PVY^{O} infection and *Ry* gene was less expressed. Lane 1, *Golden valley*; lane 2, *Winter valley*

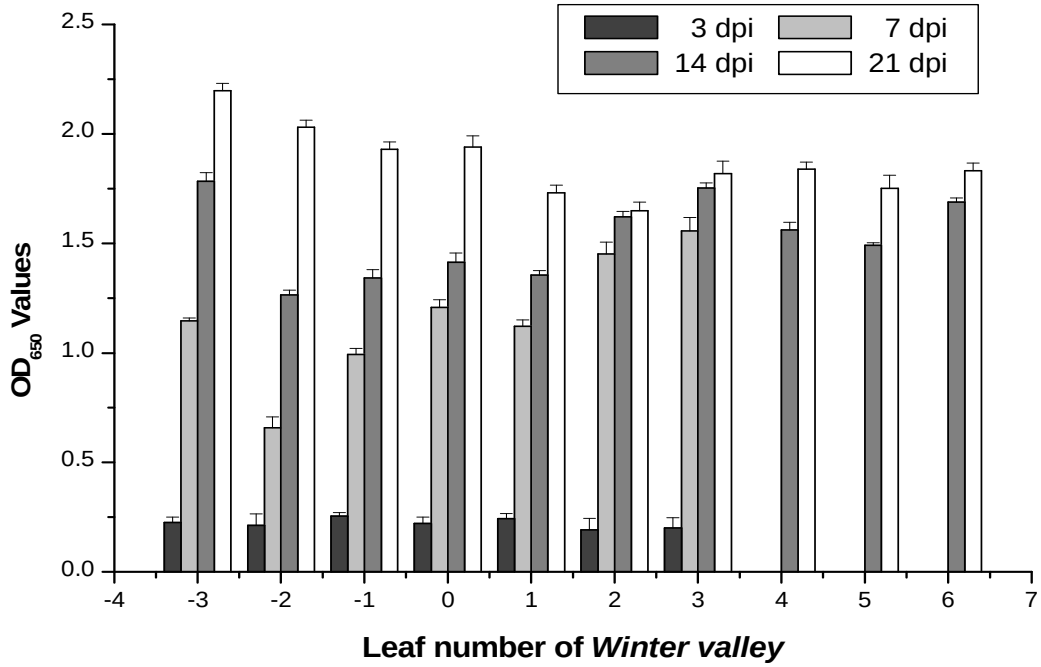

Fig. 6. DAS-ELISA OD_{650} values of leaf samples from *Winter valley*. Inoculated leaf 0, -1, -2 and -3 are the first, second and third lower leaves from the PVY^O inoculation. 1 to 6 are the first to sixth upper leaves from PVY^O inoculation. OD value was obtained higher in basal leaves after 21 dpi.

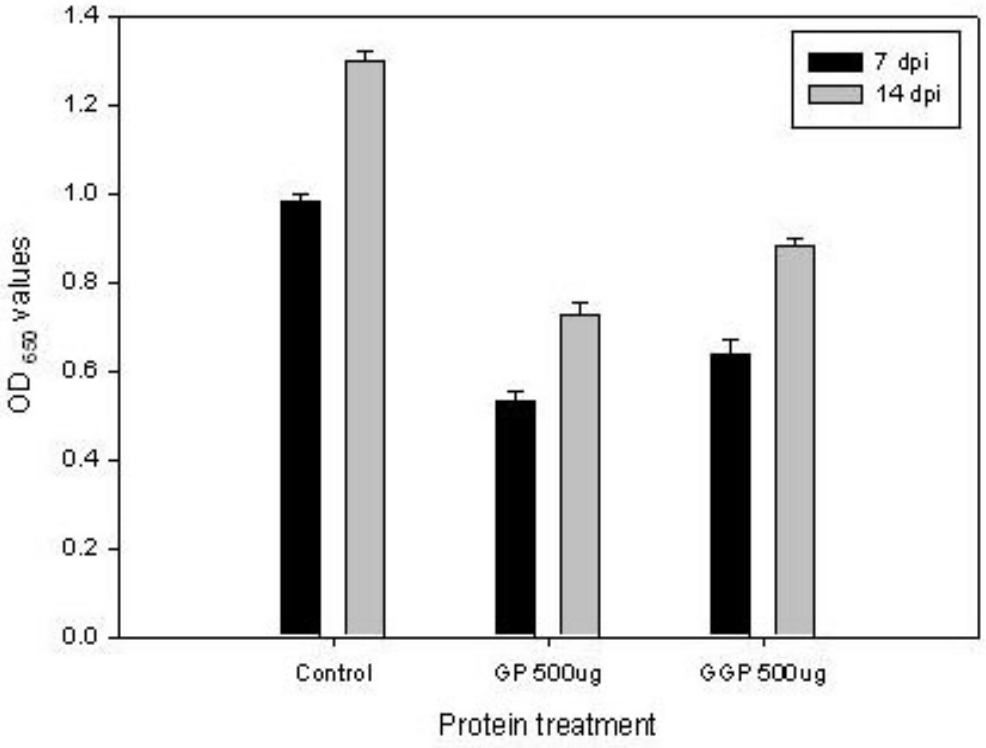

Fig. 7. Protein treatment to PVY^{O} infected potato leaves. DAS-ELISA OD_{650} values of different proteins treatment on leaves. *Golden valley* (G) and *Gogu valley* (GG) protein were applied in concentrations (µg / 5 ul) in *Winter valley* leaves. Graph showed that *Golden valley* and *Gogu valley* crude protein (500 µg) effectively reduced PVY^{O}. The test was performed after 7 dpi and 14 dpi in potato leaves first to the leaf of infection.

III – 5 - 2. G4, G11 and A3 peptides

The ELISA tests OD value result of the application of *Golden valley* and A3 peptides (10 μg) to PVY^O showed the decrease in OD value than PVY^O infected plants without peptides treatment. From this result we can suggest that *Golden valley* peptides have antiviral activity *in vivo*. A3 is well-known peptide and has been produced commercially. A3 showed strong antiviral activity against HeLa cells (Woo *et al*., 2002). A3 was treated and then PVY^O was infected to *Winter valley* leaves. PVY^O inoculated *Winter valley* was as control (Fig. 8).

III - 6. Quantitative (Real-time) PCR

When *Golden valley* leaves were infected by PVY^O, *Ry* gene was increased 2.75 times than virus-free *Golden valley* plant (Fig. 9). When *Golden valley* and *Gogu valley* proteins were treated to PVY^O, *CP* gene was decreased gradually as 48 % and 37 %. *Golden valley* derived G4, G11 and A3 peptides were treated to PVY^O, relative concentration was decreased gradually as 21 %, 49 % and 34 %. From these results we can suggest that the *Golden valley*, *Gogu valley* proteins, G4, G11 and A3 peptides have antiviral activities (Fig. 10).

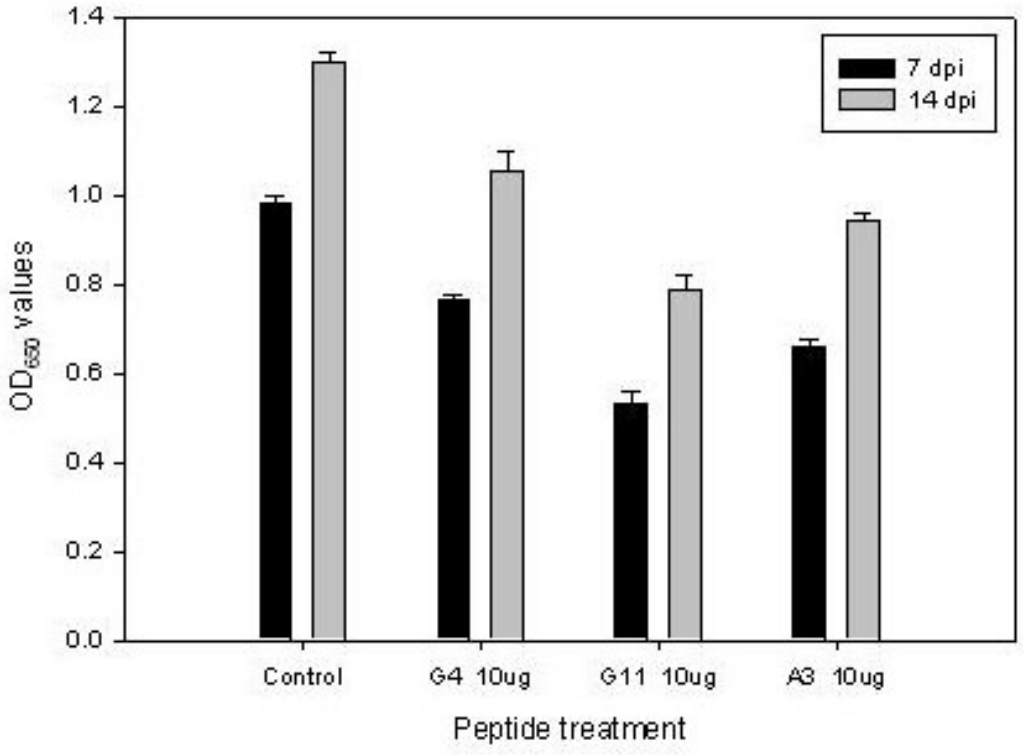

Fig. 8. The effect of peptides to PVY^{O} infected potato leaves. DAS-ELISA OD_{650} values of different peptides treatment on leaves. Peptide A3 and G (*Golden valley*) were applied in concentrations (µg / 5 µl) in *Winter valley* leaves. Graph shows peptide G4, G11 and A3 effectively reduced PVY^{O}. The test was performed after 7 dpi and 14 dpi in potato leaves first to the leaf of infection.

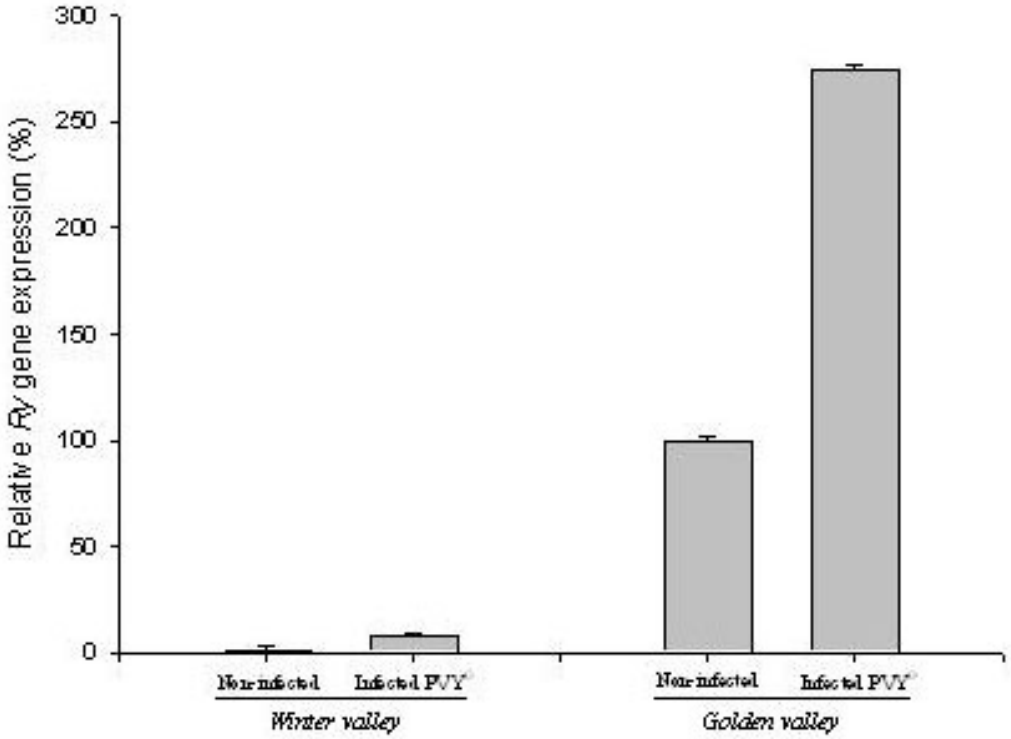

Fig. 9. **The change of *Ry* gene expression on PVY^{O} infected *Golden valley* and *Winter valley*.** Quantitative-PCR was carried out on 21 dpi PVY^{O} non-infected and infected *Winter valley* and *Golden valley. Ry* gene was 2.75 times overexpressed on PVY^{O} infected *Golden valley* than non-infected *Golden valley*.

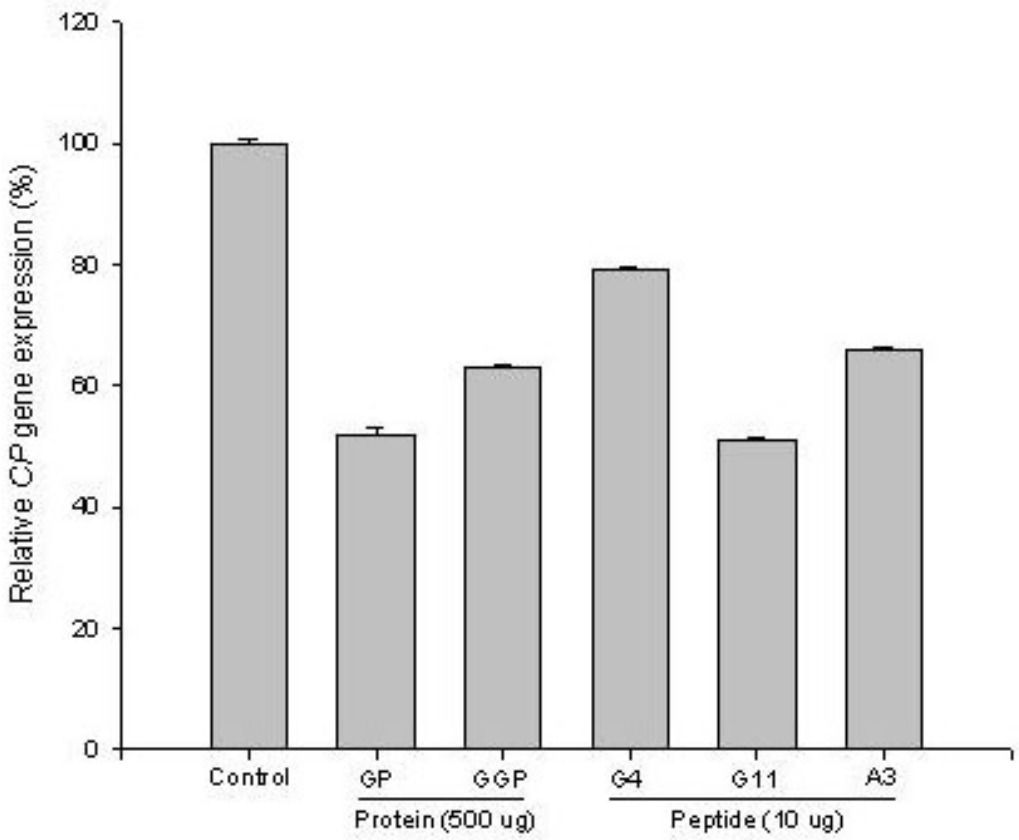

Fig. 10. The change of *CP* gene Expression by Quantitative-PCR after proteins and peptides treatment. *Winter valley* was infected PVY^{O} and 500 µg/ 5 µl protein, 10 µg/ 5 µl peptide was injected in the same leaf. After 14 dpi, expression of *CP* gene in virus infected potato cultivar terminal leaves were used for Quantitative-PCR. Control, *Winter valley* infected PVY^{O}; G, *Golden valley*; GG, *Gogu*

valley

IV. DISCUSSION

Pre-treatment of crops with chemicals is considered the only feasible means of controlling the plant disease in the agricultural fields. In recent years, the interest in environmentally friendly alternatives to the chemical pretreatments has been increased. The most widely used strategies for control of viral diseases focus on molecular breeding of genetic resistant variety. Resistance means the effect of virus infection is reduced or eliminated which ranges from tolerance of or hypersensitivity to the most durable extreme resistance (ER).

Few sources of ER provided by dominant genes exist for some potato viruses. Examples of durable resistant genes so far include *Ry* genes conferring ER to PVY. PVY is efficiently transmitted by many aphid species in a non persistent manner. PVY^{O} infected plants exhibit mosaic, leaf crinkling symptoms, not readily distinguishable from veinal necrosis. In present research, we worked with 10 valley and 2 others out of 12 potato cultivars for the screening of virus resistant potato. The development of local disease symptoms, expressed as appearance of local lesions, yellowing and dropping of inoculated leaves, as sensitivity to PVY^{O} of many cultivars. Severe local symptoms, first visible as green spots, were observed on inoculated leaves of the highly sensitive potato cultivar like *Winter Valley* between 5 and 7 dpi. In moderately sensitive cultivars like *Taebok Valley*, *Rchip Valley*, *Juice Valley* milder local symptoms were observed between 7

and 10 dpi. Systemic symptoms developed in most of these cultivars are at the same time between 9 and 14 dpi. The infection rate of inoculated leaf and next leaves were varied according to types of cultivars. The cultivar *Golden valley* was remained symptom less with PVY^O infection.

Infection method for screening of PVY^O resistant variety, it may be concluded that symptomatology is not reliable for the virus disease diagnosis. Detection by ELISA or other molecular techniques is necessary to identify PVY^O infection. Variation of symptoms in infection may be due to cultivars, time of infection, viral strain, presence of unidentified pathogens and many other factors (Jones *et al*., 1991). Three week after PVY^O infection, virus was detected by ELISA in 12 potato cultivars. All the leaf samples were taken upper leaf from the infected one. Later on, virus titre in susceptible potato cultivars reached higher level detected by ELISA (Fig. 3). In *Winter valley* ELISA result is correlated with the appearance of local symptoms. However, these results confirm the conclusion (Ravnikar *et al.*, 1995) that titre of virus, measured by ELISA in potato cultivars with different levels of sensitivity does not correlate with sensitivity, described as expression of symptoms.

In order to confirm result of ELISA and mechanical inoculation, the presence of *CP* gene was additionally checked in *Winter valley* and *Golden valley* using RT-PCR. The nature of resistance response extreme resistance or hypersensitivity, could be affected by expression of the *CP* gene (Bendahmane *et al.*, 1999). In *Winter valley* using RT-PCR proved to be a highly susceptible to the PVY^O. By comparing DAS-ELISA and RT-PCR, as expected RT-PCR proved and confirmed that *Golden valley* is resistant to PVY^O. *Ry* gene is a dominant gene which confers ER to all strains of PVY in

potato (Barker H, 1996). The molecular basis of virus resistance is not as well understood with *Ry* although the gene has been mapped to potato chromosome XI (Brigneti *et al.,* 1997; Hamalainen *et al.,* 1997; Gebhardt *et al.,* 2001) in a cluster of nucleotide binding site (NBS) and leucine-rich repeat (LRR) resistance gene homologous (Hamalainen *et al.,* 1998; Leister *et al.,* 1996). However, we report here that *Ry* mediated gene can be overexpressed in extreme resistant plants against PVY. This finding is important for understanding resistance mechanism because it indicates that *Ry* gene involves as an induce response in infected plants. However, this study suggests that the *Ry* gene confers resistance against PVY. It also suggests the heterogeneity of the *Ry* gene resulting into the double bands of different sizes in case of *Gogu valley*, while in case of *Taebok valley*, *Juice valley* and *Rchip valley* the larger band size indicate the additional bases in intercalary regions of the gene. For the verification sequencing would be valuable for confirming the bases arrangements (Fig. 4). Having exploited this approach to identify the *Ry* mediated virus resistance cultivars, there is a good prospect of molecular breeding for antiviral potato (Yi *et al.,* 2003).

Real-Time (Quantitative) PCR has engendered wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination. Quantitative-PCR has been extremely useful for studying viral agents of infectious disease and helping to clarify disputed infectious disease processes. Most of the assays presented in the literature allow an increased frequency of virus detection compared with conventional techniques, which makes the implementation of Real-Time PCR attractive to many areas of virology (Mackay *et al*., 2002). The treatment of

A3 peptide in infected potato leaves caused the reduction for PVY^{O} (Fig. 9 and 11). Similar results of dramatic increase in antibiotic activity against bacterial, fungal, and cancer cells without a hemolytic effect were obtained (Lee *et al*., 2002). And A3 peptide caused a dramatic increase in virus-cell fusion inhibitory activity (Woo *et al*., 2002). Treatment of *Golden valley* derived peptides G4 and G11 on PVY^{O} show the reduction of the viral level in the infected tissues. DAS-ELISA and Quantitative-PCR results both showed the reduction of the viral level which genetically supports the *CP* gene expression causes hypersensitivity towards PVY^{O}. The test of G4 and G11 peptides (however needs further confirmation by sequencing) to potatoes infected with PVY^{O} are found antibacterial and antifungal favoring the peptides with antibacterial and antifungal properties, however for the wide use of these proteins and peptides as biotic pesticide further test would be additive.

V. 적요

감자는 인류의 식량자원에 큰 몫을 차지하고 있는 중요한 작물이며, 식생활의 서구화로 인해 그 수요 또한 급증하고 있지만 강력한 병원성 바이러스인 감자 잎말림 바이러스, 감자바이러스 X, Y에 의하여 수확이 크게 감소된다. 감자 바이러스 Y에 감염된 식물체는 2-3주가 지난 후 모자이크 반점, 황화현상, 잎의 괴사, 엽맥의 투명화등의 형태학적 변화를 보이며 15~75 %의 수확량 감소를 가져온다. 본연구는 ELISA (면역학적인 기법을 이용한 단백질의 분석방법)와 RT-PCR (역전사-중합효소연쇄반응)을 이용하여 골든밸리, 썸머밸리, 윈터밸리, 태복밸리 등 12종의 감자에서 바이러스에 저항성이 높은 감자를 선별하였다. 그리고 *Ry* (PVY 저항성 유전자)를 이용하여 RT-PCR 한 결과 PVY^{O}에 감염되지 않았을 때보다 감염된 골든밸리에서 많은 양이 발현되었다. Quantitative-PCR (실시간-중합효소연쇄반응)을 통해 분석한 결과 골든밸리로부터 추출한 단백질 혹은 펩타이드(G4, G11), A3 펩타이드를 PVY^{O}를 처리한 윈터밸리에서보다 바이러스의 감염도가 줄어든 것을 볼 수 있었다. 이러한 결과를 기반으로 항바이러스성 감자종으로부터 추출한 펩타이드를 감자를 포함한 가지과 식물에 적용함으로써 식물체에 존재하는 바이러스에 대한 저항력을 높이며, 화학농약을 대체할 수 있는 생물농약을 개발할 수 있다.

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