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碩士學位論文

Expression and characterization of virus resistant gene in *Solanum tuberosum* L. *cv. Golden valley*

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골든밸리감자에서 바이러스 저항성 유전자의 발현 및 분석

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ABBREVIATIONS

BSA	Bovine serum albumin
CC domain	Coiled coil domain
CP	Coat (Capsid) protein
CIP	Calf intestinal alkaline phosphatase
DAS	Double-antibody sandwich
DTT	Dithiothreitol
dpi	Days post inoculation
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Extreme resistance
HC-pro	Helper component proteinase
HR	Hypersenstive resistance
LRR	Leucine-rich repeat
NBS	Nucleotide-binding site
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

ABSTRACT

Expression and Characterization of Virus Resistant Gene in Potato

(감자에서 바이러스 저항성 유전자의 발현 및 분석)

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Potato Virus Y (PVY), the type member of the genus potyvirus in the family Potyviridae is a widespread virus leading to severe damage in Solanaceae, which was loss crop yield up to 75% in Potato (*Solanum tuberosum* **L.**). PVY infected plants shows several symptoms like mosaic, yellowing, necrosis, mottle, vein clearing, vein bending and misshapen in tubers. Plants carrying the *Ry* gene are resistance to a number of potiviruses. Previously, we identified that the potato cv. Golden valley has rich in *Ry* gene overexpression. We isolated crude protein form cv. Golden valley and applied to the most virus-susceptible potato cv. Winter valley among the twelve Korean cultivars studied. Real-time PCR and ELISA results showed that in vivo application of Golden protein to cv. Winter valley could reduce the rate of PVY accumulation significantly. Analysis of *Ry* gene sequence was showed that there were about 91% sequence homologs between *Solanum tuberosum subsp. andigena* and *Solanum tuberosum* **L.** *cv. Golden valley*. Our second phase research work is going on to develop virus resistant potato cultivars by introducing, *Ry* gene and bio-pesticides for other legume crops simultaneously.

INTRODUCTION

Potatoes can be infected by a large number of viruses(Valkonen, 1994) and are vulnerable to virus infections because they allow the transfer of viruses from one vegetative generation to the next(Hinrichs *et al.*, 1998). Potato virus Y (PVY), belonging to the virus group Potyviridae, is one of the most damaging plant virus economically affecting to potato(*Solanum tuberosum* **L.**)(Shukla *et al.*, 1994). PVY is transmitted by means of mechanical, vector or environmental in all areas where potatoes are grown and yield losses vary from 30 to 80% depending upon potato cultivars and virus strain(Fang *et al.*, 2005; Hull, 1984; Debokx and Huttinga, 1981). Potato strains are commonly subdivided into three main strains PVY^{O}, PVY^{C} and PVY^{N}. PVY^{O} is the common or ordinary strain in most potato producing countries (Jones, 1990). The disease is characterized with primary symptoms of necrosis, mottling, and yellowing of leaves that may cause premature death. The most widely used strategies for control of virus diseases focus on characterization of virus resistance potato cultivars. Resistance describes here is the effect of virus infection is reduced or eliminated which ranges from tolerance of or hypersensitivity to the most durable extreme resistance or immunity. Few sources of extreme resistance provided by dominant genes exist for some potato viruses. Examples of durable resistance genes so far include a dominant gene *Ry* gene which confers extreme resistance (ER) to all strains of potato virus Y in potato(Sabina *et al.*, 2002; Valkonen *et al.*, 1998; Valkonen *et al.*, 1997; Adams *et al.*, 1986; Dykstra, 1939). The *Ry* mediated resistant plants do not develop visible symptoms when challenged with the virus, virus accumulation cannot be detected by ELISA and the resistance is active at the protoplast level(Adams *et al.*, 1986; Baker *et al.*, 1984).

Korean potato cultivars vary from highly sensitive to tolerant in their

sensitivity to infection with PVY^O. Only a few potato cultivars, like Golden Valley was found to be resistant to PVY^O. Different methods have been used to characterize the PVY^O resistance potato(Fang *et al.*, 2005; Nei *et al.*, 2001; Myslik *et al.*, 2001; Andre *et al.*, 2001). The most simple involve scoring symptoms observed on PVY^O inoculated on first leaves of plant. If the systematic symptoms appeared, it was supposed the virus spread from the inoculated leaves to other parts of the plant. However, this method was not applicable to all potato cultivars like in case of PVY^O tolerant and semi tolerant plant. Several molecular methods have been developed to identify the PVY^O resistant potato. Therefore, it was necessary to apply specific methods like molecular and serological, targeting to nucleic acid and virus particles.

Here we describe an alternative approach for the characterization of PVY resistant potato based on ELISA and RT-PCR. Coat protein (*CP*) gene is involved in virus spreading 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. Results of ELISA and *CP* gene from RT-PCR were cross checked with TEM and PVY^O inoculated phenotypes. The aim was to screen the PVY^O resistant potato from Korean potato cultivars. Our screening conferred that the potato Winter Valley was highly susceptible to PVY^O and the Golden Valley was extremely resistant. The results of this study may serve the design and interpretation of molecular research on virus resistant plant screening and development. It will facilitate selection of resistant cultivars obtained by either classical or molecular techniques.

MATERIALS AND METHODS

1. Plant materials and Growth condition

All the potato tuber samples (32 different Korean cultivars) were obtained from Potato valley, Korea. All the samples were healthy and virus free. First, the tubers were propagated, on autoclaved soil (Mix 5 Soil, Sun Grow Horticulture, Canada), in growth room and when plant became 4 to 6 ㎝ tall and all plants samples were aseptically transferred in vitro via single node cutting in 3% Sucrose, Murashige and Skoog (MS) medium. In vitro propagated plants were subcultured by transferring nodal segments to fresh medium every 4 weeks until the experiment performed. Potato plants were grown in a growth room, at 23±1℃, under a 16-h photoperiod

2. Virus innoculation

The PVY virus ampoule was obtained from Plant Virus Genbank, Seoul Women's University, Korea. The obtained sample was immediately frozen in liquid nitrogen, and then finely powdered in a mortar and pestle. The powdered, frozen tissues were thawed in 1:200 (W/V) 0.1 M sodium phosphate buffer (pH 7.0). Silicon carbide (400 mesh) dusted potato leaves were mechanically inoculated with prepared PVY^{O} inoculi. After three weeks of PVY^{O} inoculation, young emerging apical leaflets of each potato plants were checked for PVY^{O} infection.

3. ELISA analysis for detection from PVY^{O} infected potato varieties

3-1. 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 ㎚ by using a microplate reader (Molecular Devices Co., Sunnyvale, CA, USA) as described by Park *et al.* (2004). We have used 10 ㎍/100 ㎕ proteins for ELISA test.

3-2. ELISA test

The direct Double-Antibody Sandwich ELISA method (DAS-ELISA) (Agdia, Inc., Elkhart, IN) was used, according to instructions provided by Agdia Inc. All reagents were obtained commercially from Agdia, Inc. PVY infected leaf samples were extracted in a 1:10 dilution of the appropriate buffer (Agdia general extractuib buffer), microcentrifuged at 12,000 rpm for 5 min and then added to microtiter plates. The samples were observed spectrophotometrically at 405 ㎚ in an ELISA Reader (Bio-Rad, USA). All assays were performed in triplicates.

4. Expression pattern of *CP* and *Ry* gene by RT-PCR

4-1. Total RNA extraction

Total RNA was extracted from PVY infected leaf using TRIzol reagent (Life Technologies). Leaf tissues were frozen in liquid nitrogen and ground into autoclaved mortar and pestle. Total RNA was extracted from a sample of finely grinded homogenous powdered tissue (~100 ㎎) according to the manufacturer's instructions. Total RNA was then eluted in 20 ㎕ of RNase-free water. The RNA concentration was quantified by measuring the absorbance at 260 ㎚ using spectrophotometer (Bio-Red,

Smart Spec TM 3000).

4-2. cDNA synthesis

cDNA was synthesized using Superscript II reverse-transcriptase (Life Technologies). 2 ㎍ of total RNA and 1 ㎕ of the oligo dT (500 ㎍/㎖) were mixed in a reaction tube and heated at 65℃ for 10 min to inactivate the sample and then quick chilled on ice. 5x first strand buffer and 0.1 M DTT were added, and the mixed contents of the tube gently incubated at 42℃ for 2 min. 1 ㎕ (200 units) of Superscript II was added into the tube, incubated at 42℃ for 50 min and the reaction stopped by heating at 70℃ for 15 min. The synthesized cDNA was ready for RT-PCR and rest is stored at -20℃ for further use.

4-3. RT-PCR amplification

For amplification of the *CP* gene, the forward primer, 5'-AGGCACATCT GGGACACATACTGTGCCGA-3', known as YCPF, located in the Coat Protein gene position **8680-8708**, and reverse primer 5'-TGACTCCAAGT AGAGTATGCATACTTGGA-3', known as YCFR, located downstream at position **9336-9365**, according to the NCBI accession number **NC_001616** were used. The *CP* gene was amplified by PCR, with a denaturation of 5 min at 94° C; 35 cycles at 94° C for 30 s; 57° C for 30 s and 72° C for 1 min, with a final extension of 5 min at 72° C. Similarly, the *Ry* gene was amplified by PCR, with a denaturation of 5 min at 94° C; 35 cycles at 94° C for 30 s; 52° C for 30 s and 72° C for 7 min, with a final extension of 4 min at 72° C. Similarly, For amplification of the *Ry* gene, the forward and reverse primers, 5'-TTGAAGAAAGTTCTAGTTGTGCT -3', known as RyRF, and 5'-TGAAATGCTTGTCTCTCGTTGTTG-3', known as RyRR, from the NCBI accession number **AJ300266**, were used.

All primers were selected by computer analysis using the prime3 program. A 20 ㎕ RT-PCR reaction mixture, contained 0.25 mM of dNTPs, 0.5 ㎛M of both primers, 1xTaq buffer, 1 ㎕ cDNA and 1 unit Taq DNA polymerase. The *Ry* gene was amplified by PCR, with a denaturation of 5 min at 94℃; 35 cycles at 94℃ for 30 s; 52℃ for 30 s and 72℃ for 7 min, with a final extension of 4 min at 72℃.

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

Gene name	Forward primer	Reverse primer	Fragment (bp)
CP gene (RT-PCR)	YCPF : 5'-AGGCACATCTGGGACACATACTGTGCCGA-3'	YCPR : 5'-TGACTCCAAGTAGAGTATGCATACTTGGA-3'	690
Ry gene (RT-PCR)	RyRF : 5'-TTGAAGAAAGTTCTAGTTGTGCT-3'	RyRR : 5'-TGAAATGCTTGTCTCTCGTTGTTG-3'	154
β-actin	ActinF : 5'-GGCGATGAAGCTCAATCCAAACG-3'	ActinR : 5'-GGTCACGACCAGCAAGATCAAGACG-3'	495

5. Application of $Protein_{golden}$

5-1. $Protein_{golden}$ preparation.

The crude protein was isolated, following the frozen leaf material from the *Ry* gene rich potato was ground to homogenous powder in liquid nitrogen with mortar and pestle. The homogenate was thawed and incubated with extraction buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, and 2% 2-mercaptoethanol at 4℃ for an hour. The mixture was clarified by centrifugation at 12,000 rpm for 15 min at 4℃. The supernatant was pooled and freeze-dried to obtain the concentrate dry powder. Obtained protein powder was dissolved in autoclaved distilled water before use.

5-2. $Protein_{golden}$ treatment

Potato highly susceptible to PVY^O infection (cv. *Winter valley*) was grown as described above were used for this test. 500 ㎍ of protein extracted from leaves of cv. *Golden valley* were applied separately by cotton swab on the dorsal side of healthy leaf of 3 weeks old plant. After 10 minutes of protein treatment, the plants were infected with PVY^O virus as described above. PVY^O infected cv. Winter valley was used as a control.

6. Expression pattern of *CP* gene and *Ry* gene against PVY^O using Quantitative(Real-time) PCR

Each 20 ㎕ reaction mixture contained the following ingredients: QuantiTect SYBR Green PCR Master Mix (HotStarTaq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTPs including dUTP for optional uracil-N-glycosylase treatment, SYBR Green I, and ROX a passive reference dye), 5 pmol of each primer, 200 ng template cDNA and RNase -free water were added. All the quantitative PCRs were performed 15

min polymerase activation at 95℃ and 40 cycles of denaturation at 95℃ for 10 sec, annealing at 54℃ for 20 sec, extension at 72℃ for 30 sec and a final extension at 72℃ for 5 min in a fluorometric thermal cycler (Rotor-Gene 3000, Corbett, Australia). Real-time PCR assay was performed in triplicate. The expressed relative concentrations were analyzed using Rotor-Gene Real-Time Software 6.0.

7. Cloning of *Ry*$_{golden}$ gene

7-1. cDNA synthesis of *Ry*$_{golden}$ gene

The two fragments of *Ry* gene were amplified by using primers, Forward(RyF10) 5'-ATCCCGGGATGGCATCATCATCTTCTTC-3', Reverse (RyIR) 5'-AAGCATGCAATATCTAGAAATATCTCTTGA-3', Forward(RyIF) 5'-TTGCATGCTTCTTAAGAGGGAGAAAACAAA-3', Reverse (RyR10) 5'-TACCCGGGCTCACATTGTCAACATCTTCAGT-3'. A 20 ㎕ PCR reaction mixture contained 0.25 mM of dNTPs, 0.5 ㎛M of both primers, 1xTaq buffer, 1 ㎕ cDNA and 1 unit Taq DNA polymerase. The reaction mixtures carried out PCR using the Thermocycler with cycling conditions : 94℃ for 5min; 35 cycles of denaturation (94℃ for 30 s), annealing (50℃ for 1 min) and extension (72℃ for 5 min). The reactions were terminated with an extension step at 72℃ for 5 min. (Figure 1)

7-2. Vector(pUC19) and *Ry*$_{golden}$ gene preparation

pUC19 vector (≒2.3 kb) and inserts were digested with restriction endonuclease, XmaI and SphI. A 20 ㎕ digestion mixture contained 1x NEB buffer4, 1x BSA, restriction endonucleases (XmaI and SphI), The mixtures were incubated at 37℃ for 48 h. The pUC19 vector was added a 1 ㎕ of CIP (New England Biolabs). Then, the mixture was incubated at 37℃ for 1 h. Each mixtures were heat-inactivated at 65℃ for 20 min. (Figure 2)

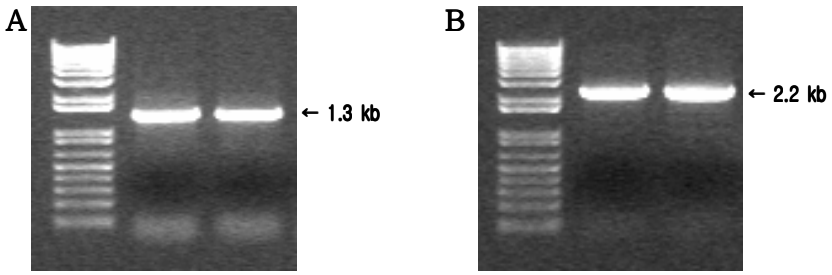


Figure 1. The two fragments of *Ry* gene left (1.3 kb) and Right (2.2 kb) were amplified by using primers, **A.** Forward (RyF10) 5'-ATCCCG GGATGGCATCATCATCTTCTTC-3', Reverse (RyIR) 5'-AAGCA TGCAATATCTAGAAATATCTCTTGA-3', **B.** Forward (RyIF) 5'-T TGCATGCTTCTTAAGAGGGAGAAAACAAA-3', Reverse (RyR10) 5'-TACCCGGGCTCACATTGTCAACATCTTCAGT-3'.

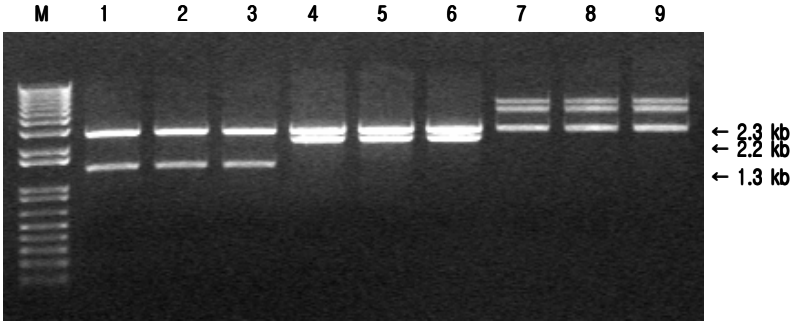


Figure 2. pUC19 vector and *Ry* gene fragments were digested with XmaI, SphI. Lane 2~4 : The front site digested *Ry* gene fragment (1.3 kb), Lane 5~7 : The back site digested *Ry* gene fragment (2.2 kb), Lane 8~10 : The digested pUC19 vector (2.3 kb).

7-3. Vector(pUC19) and *Ry*$_{golden}$ gene ligation and transformation

The digested vector (pUC19) and insert were ligated with T4 DNA ligase (New England Biolabs). A 20 ㎕ mixture for ligation was containing 1x ligation buffer, T4 DNA ligase, pUC19 vector and two insert fragment. The mixtures were incubated at 16℃ for 24 h. After ligation, the mixtures were heat-inactivated at 65℃ for 10 min. The mixtures were transformed in DH5α competent cell by heat-shock method.

The constructs were screened by cell PCR. pUC19 vector and the first inserted DNA fragment construct was amplified by using primers, Forward (pUC19R) 5'-CCATTCGCCATTCAGGCTGCGCAACT-3', Reverse (RyRR) 5'-TGAAATGCTTGTCTCTCGTTGTTG-3' and pUC19 vector and the second inserted DNA fragment of *Ry* gene construct was amplified by using primers, Forward (pUC19F) 5'-TGAGTTAGCTCACTCATTAGGCA-3', Reverse (RyF) 5'-TCTAGTCTTACAGAGCTAGATTTGAGT-3'. A 20 ㎕ PCR reaction mixture contained 0.25 mM of dNTPs, 0.5 ㎛M of both primers, 1x Taq buffer, 1 ㎕ cDNA and 1 unit Taq DNA polymerase. The reaction mixtures carried out PCR using the Thermocycler with cycling conditions : 94℃ for 5 min; 35 cycles of denaturation (94℃ for 30 s), annealing (55℃ for 30 s) and extension (72℃ for 3 min). The reactions were terminated with an extension step at 72℃ for 5 min. (Figure 3)

7-4. Plant expression vector(pBI121) and *Ry*$_{golden}$ gene preparation

vector pBI121 (≒14.7kb) was digested with restriction endonuclease, XmaI. A 20 ㎕ digestion mixture containing 1x NEB buffer 4, 1x BSA, restriction endonucleases (XmaI). Each of pBI121 and The two fragments of XmaI and SphI-digested *Ry* gene mixtures were incubated at 37℃ for 48 h. The pUC19 vector was added a 1 ㎕ of CIP (New England Biolabs). Then, the mixture was incubated at 37℃ for 1 h. Each mixtures were heat-inactivated at 65℃ for 20 min. (Figure 4)

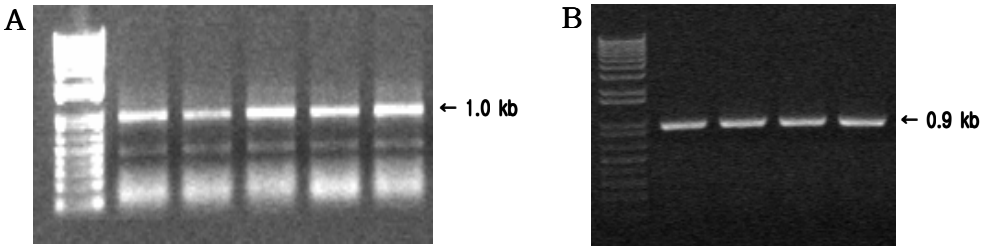


Figure 3. To make sure whether DNA was inserted or not, we screened the construct by PCR. **A.** pUC19 vector and the first inserted DNA fragment construct was amplified by using primers, Forward (pUC 19R) 5'-CCATTCGCCATTCAGGCTGCGCAACT-3', Reverse (RyRR) 5'-TGAAATGCTTGTCTCTCGTTGTTG-3'. **B.** pUC19 vector and the second inserted DNA fragment of *Ry* gene construct was amplified by using primers, Forward (pUC19F) 5'-TGAGTTAGCTC ACTCATTAGGCA-3', Reverse (RyF) 5'-TCTAGTCTTACAGAGCT AGATTTGAGT-3'.

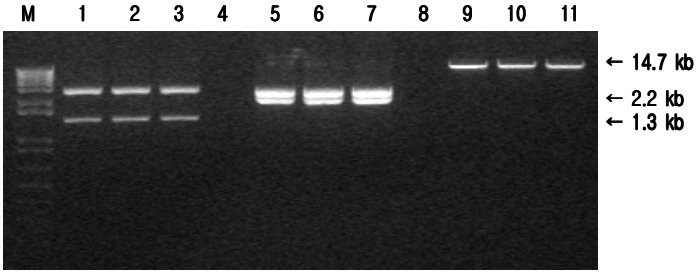


Figure 4. pBI121 vector and pUC19 vector containing the *Ry* gene fragments were digested with restriction endonucleases. Lane 2~4 : The front site digested pUC19 vector contained *Ry* gene fragment (1.3 kb) (digested with XmaI and SphI), Lane 6~8 : The back site digested pUC19 vector contained *Ry* gene fragment (2.2 kb) (digested with XmaI and SphI), Lane 9~11 : The digested pBI121 vector (14.7 kb) with restriction endonuclease (XmaI).

7-5. Vector(pBI121) and *Ry*$_{golden}$ gene ligation and trnasformation

The digested vector (pBI121) and inserts were ligated with T4 DNA ligase (New England Biolabs). A 20 ㎕ mixture for ligation was contained 1x ligation buffer, T4 DNA ligase, pUC19 vector and two different insert fragment. The mixtures were incubated at 16℃ for 24 h. After ligation, the mixtures were heat-inactivated at 65℃ for 10 min. The mixtures were transformed in DH5α competent cell using heat-shock method. (Figure 5)

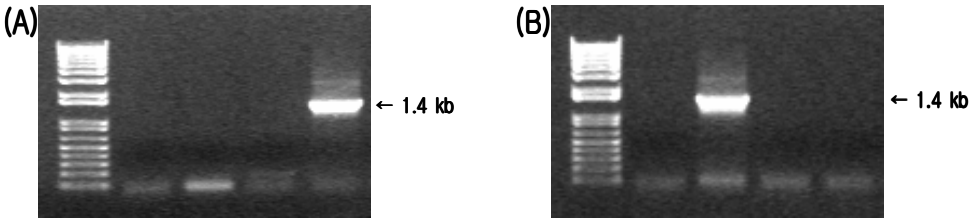


Figure 5. To make sure whether introduced DNA was present or not, we check the construct by PCR. **A.** pBI121 vector and the sense line of *Ry* gene construct was amplified by using primers, Forward (RyF) 5'-TCTAGTCTTACAGAGCTAGATTTGAGT-3', Reverse (pBI121R) 5'-TCGCGATCCAGACTGAATGCCCACA-3'. **B.** pUC19 vector and the back site of *Ry* gene construct was amplified by using primers, Forward (RyF) 5'-TCTAGTCTTACAGAGCTAGATT TGAGT-3', Reverse (pBI121F) 5'-TCCCACTATCCTTCGCAAGAC CCTTCCTCT -3'.

RESULTS AND DISCUSSION

1. The pattern of PVY^O virus infection between potato lines

PVY^O induces mild to severe mosaic, mottling or yellowing, leaf drop and premature death, and can cause stem necrosis in potato. First, we screened on the basis of leaf infection morphology. 32 Korean potato cultivars were inoculated with PVY^O at the six to eight leaf stage (approximately 3 week plant). Six to seven days post-inoculation (d.p.i.), necrotic streaks appeared along the veins of the abaxial surface of PVY^O-inoculated leaflets of susceptible plant, in addition to small necrotic lesions on the interveinal region, were also observed. The mosaic symptoms increased rapidly until twenty-one d.p.i. during the study period of 45 days. On the basis of leaf infection morphology we categorized the potato varieties as highly susceptible, Susceptible and Resistant to the PVY (Example table 2). Then for our further purpose we just choose the highly susceptible and resistant cultivar for the further study. (Figure 6)

There are two major types of resistance to PVY in potatoes; extreme resistance (ER) and hypersensitive resistance (HR). A hypersensitive response is characterized by fast and localized cell death at the site of attack by pathogens in the most incompatible interactions controlled by disease resistance genes. ER strongly suppresses virus accumulation in infected cell, with no visible symptoms or detectable amounts of PVY^O observed in inoculated plants.

Few sources of extreme resistance are provided by dominant genes for some potato viruses. An example of a durable resistance gene is the *Ry* gene, which confers extreme resistance to PVY^O. PVY^O is efficiently transmitted by many aphid species in a non persistent manner. PVY^O infected plants exhibit mosaic, leaf crinkling symptoms, which are not

readily distinguishable from veinal necrosis. In this work, 32 different Korean potato cultivars were analyzed for screening of virus resistant potatoes. The development of local disease symptoms, expressed as the appearance of local lesions, yellowing and dropping of inoculated leaves, suggestive of sensitivity to PVY^{O} occurred in many cultivars. (Figure 6) Severe local symptoms, first visible as green spots, were observed on inoculated leaves of the highly sensitive potato cultivar, such as Winter valley, between 5 and 7 days after inoculation. In moderately sensitive cultivars, such as Taebok valley, Rchip valley, and Juice valley, milder local symptoms were observed between 7 and 10 dpi. Systemic symptoms developed in most of these cultivars at the same time; between 9 and 14 days after inoculation. The infection rate of inoculated the leaf and the adjacent leaves varied according to the types of cultivar. (Table 2) The cultivar Golden valley remained symptomless when inoculated with the PVY^{O}.

PVY^{O} infection rate									
	Early	Golden	Gogu	Juice	Purple	Rchip	Summer	Taebok	Winter
Infected leaf	++	−	+	++	+++	++	+	+	+++
Next to the infected	++	−	++	+	++	−	−	+	+++
+++ : >75% leaf area infected, ++ : 50% leaf area infected, + : <25% leaf area infected, − : not infected									

Table 2. The level of necrosis on the PVY^{O} infected leaf and those above the infection in different potato cultivars after three weeks of PVY^{O} infection.

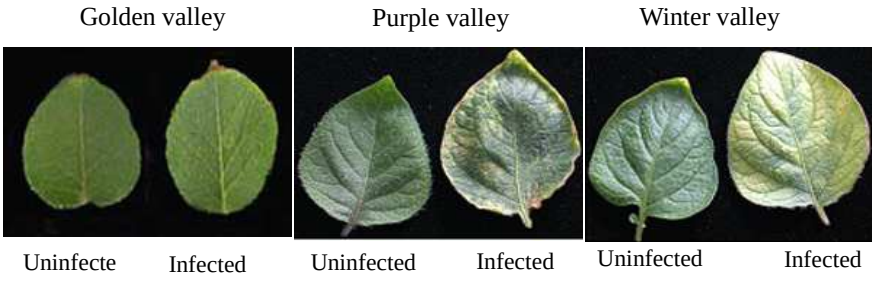


Figure 6. All the PVY^{O} infected leaves (right) are just above leaf from the inoculated ones were taken from Golden valley, Purple valley and Winter valley cultivars. 21 days after inoculating the lower leaves with an extract of PVY^{O}. The uninfected leaf (left of each) is also same aged PVY^{O} uninfected plant. Several local lesions developed on the PVY^{O} infected Purple valley and Winter valley.

2. Selection of virus resistant potato using DAS-ELISA

According to the infection leaf morphology, we selected 11 cultivars on the basis of susceptible and resistance and were performed ELISA test. Two leaves per plant, of a total of 5 plants per cultivar, were inoculated therein triplicate experiments. 3 weeks post-inoculation, two upper leaves from the inoculated plant were tested for PVY^{O} infection using DAS-ELISA. Sap of 0.1 M Sodium phosphate buffer (pH 7.0) injected Winter valley was used in the ELISA as negative controls. The extracts from PVY^{O} infected *Burley21* was used in different concentrations as a positive control. (Figure 7B) The absorbance at 405nm (optical density, OD) was measured at intervals of 20 min, and 1 and 2 hours after incubation with the substrate. Data obtained from the OD readings after 2 h of incubation in the substrate were taken for further analysis. The significant differences ($P<0.05$) between the mean values was determined using the Student's t-test. The detection of the virus in plants tested varied according to the level of the PVY^{O} found in the symptomatic leaves using the ELISA test. (Figure 7A) In contrast, no PVY^{O} infection was detected in either the infected or non-infected leaves of Golden valley. This study shows that high levels of resistance to PVY^{O} were expressed in Golden valley. The OD value of the PVY^{O} infected Golden valley was found to be similar to that of the negative control. (Figure 7B) The results of this study confirm that Golden valley and Winter valley as a virus resistant and a highly susceptible, respectively, varieties of potato among those studied.

As a method for infection screening of PVY^{O} resistant varieties, it may be concluded that symptomatology is not reliable for the diagnosis of a viral disease. Detection by ELISA or other molecular techniques are necessary for the identification of PVY^{O} infection. In the context of time and economic constraints, 21 of the 32 cultivars had to be omitted from

the population for the DAS-ELISA test, as they were detected as being identical in the phenotype analysis. Variations in the symptoms of infection may be due to the cultivars used, the time of infection, the viral strain, presence of unidentified pathogens and many other factors. Three weeks after PVY^{O} infection, the virus was detected by ELISA in eleven potato cultivars. All the samples were taken from the upper infected leaves. Later on, the virus titres in the susceptible potato cultivars reached higher levels, as detected by ELISA. (Figure 7) In Winter valley, the ELISA result was correlated with the appearance of local symptoms. However, this result confirms that the titre of the virus in the potato cultivars, as measured by ELISA, had different levels of sensitivity, which did not correlate with the sensitivity described by the expression of symptoms. (Table 2) The ELISA method was unable to detect the virus in any of the infected Golden valley plants.

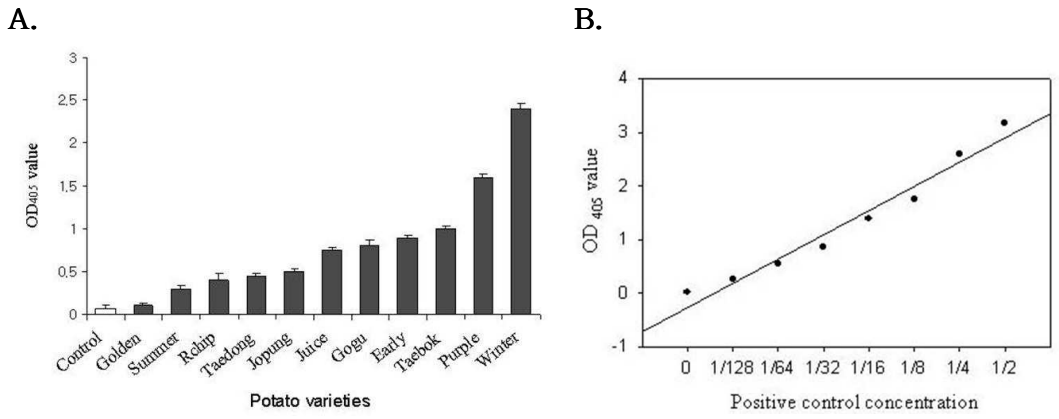


Figure 7. DAS-ELISA method for identification of PVY infection. A. Accumulation of PVY^{O} in different cultivar of potatoes. In DAS-ELISA OD values were obtained after 2 h of incubation in the substrate; in triplicate. Each variety is the average of five samples. Standard errors are indicated by the error bars, with significant difference at $P<0.05$. Control is the mock inoculation of 0.1M Sodium phosphate buffer (pH 7.0) only in Winter valley.. B. Positive Control: The sap of PVY^{O} infected *Burley21* leaves was extracted and diluted with 0.1M sodium phosphate buffer (pH 7.0) in different concentrations (v/v) and measured OD_{405} in ELISA Reader.

3. Characterization of virus resistant and susceptible cultivars

3-1. Expression of *CP* gene

The Coat protein (*CP*) gene is used in the assembly of virus particles and controls virus transmission by aphids. Recently, coat protein was also found to be involved in the spread of a virus by cell-to-cell movement. It is possible that the *CP* gene is able to increase the SEL (size exclusion limit) of plasmodesmata and facilitate cell-to-cell movement of viral RNA. In order to confirm the DAS-ELISA result further, RT-PCR on *CP* gene was carried out separately in PVY^{O} inoculated leaves. The upper young leaves of 3-week post inoculated potato leaves were tested for expression of the *CP* gene. When the PVY^{O} coat protein region was amplified, using the two specific primers, YCPF and YCPR, a band of 690 bp in size, as expected, were observed strongly on cv. Early valley, cv. Winter valley, cv. Taebok valley. (Figure 8) This result conferred that Golden valley was extremely resistant to PVY^{O}.

3-2. Expression of *Ry* gene

Similarly, *Ry* gene was also amplified on using primers forward RyRF and reverse RyRR. In many cultivars, no *Ry* gene could be detected (data not shown). However, a 154 bp band, corresponding to the expected size of the sequence included between both primers, was observed in cv. Golden valley strongly. (Figure 8)

We detected the mRNA expression level of *Ry* gene and identified the types of resistance. Sometimes, it is also accepted that the result of RT-PCR for virus detection depends on the quality of cDNA, which is determined by many factors. However, from this study, it is suggested that ELISA is a more sensitive tool for virus detection, but RT-PCR could

be used as a complementary method, and conforming the cultivars is either virus susceptible or resistant.

In order to confirm the results of the ELISA and mechanical inoculation, the presence of the *CP* gene was additionally checked in Winter valley and Golden valley using RT-PCR. The nature of the resistance response; extreme resistance or hypersensitivity, could be affected by the expression of the coat protein (*CP*) gene. From the RT-PCR, Winter valley was shown to be highly susceptible to the PVY^{O}, and by comparing the DAS-ELISA and RT-PCR, as expected, the RT-PCR proved and confirmed that Golden valley was resistant to PVY^{O}.

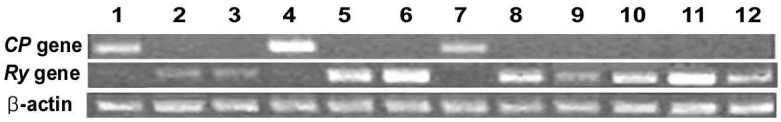


Figure 8. Screening of virus resistant potato using RT-PCR. Comparative band intensity of virus resistant (*Ry*) gene and coat protein (*CP*) gene were detected on RT-PCR amplification using specific primers as described in materials and methods. cDNAs were synthesized separately from RNA isolated from different cultivars of the PVY^{O} infected potato. Lane 1. cv. Early valley, Lane 2. cv. Gogu valley, Lane 3. cv. Purple valley, Lane 4. cv. Winter valley, Lane 5. cv. Taedong valley, Lane 6. cv. Summer valley, Lane 7. cv. Taebok valley, Lane 8. cv. Jopung valley, Lane 9. cv. Juice valley, Lane 10. cv. Rchip valley, Lane 11. cv. Golden valley, Lane 12. cv. Superior valley.

4. *Ry* gene expression in potato lines

To understand the up-regulation of (*Ry*) gene, we examined the mRNA expression level of *Ry* gene using real-time PCR on RNA extracted from the leaves of PVY^{O} infected cv. Golden valley and Winter valley. The relative expression level of the *Ry* gene in PVY^{O} infected cv. Golden valley was found to be approximately 3 times higher on average than that of the uninfected control plant. (Figure 9)

Quantization of host-specific resistant gene is very important to know the host pathogen interactions. Recently, real-time fluorescent PCR is a most frequently used technique for the detection and quantification of viral particles in different parts of the plant. We found that *Ry* gene of Golden valley was highly expressed against PVY^{O} infection. (Figure 9) We identified *Ry* gene-rich cultivar and further extracted the protein from its leaves. Plant-resistant (R) gene encoded protein contains an N-terminal coiled-coil (CC) domain, a central nucleotide-binding site (NBS) domain, and a C-terminal leucine-rich repeat (LRR) domain. CC-NBS-LRR recognizes specific pathogen-derived products and initiates resistant response.

5. The virus resistant effect of $Protein_{golden}$

We isolated the crude protein from highly and moderately resistant cv. Golden valley, respectively, and treated the Winter valley and infected with PVY^{O}. After the 14 dpi, the treated leaves were harvested, and we examined the PVY^{O} sensitivities using ELISA. The application of each 500 ug of Golden protein reduced the PVY^{O} accumulation on average 50%. (Figure 10) We found that protein extracted form the Golden valley exerts an antiviral effect against PVY^{O} infection. There is indirect evidence that the LRR may contribute to signaling as well as recognition

specificity. However, it is generally accepted that the resistance (R) proteins mediate elicitor recognition and activate downstream signaling responses, leading to the disease resistance. Thus, further investigation is needed to understand the signaling response against the virus pathogen in plant.

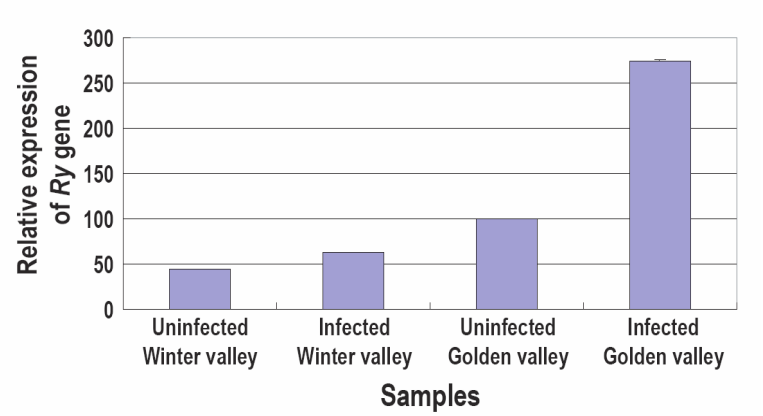


Figure 9. *Ry* gene is expressed 2.6 times more, after the PVY^{O} infection in virus resistant cv. Golden valley. The relative expression of *Ry* gene was measured on PVY^{O} infected and non-infected potato cultivars Golden valley and Winter valley both by using quantitative Real-Time PCR (Corbet 3000, Australia). Amounts of cDNAs were calibrated using actin as reference. Data presented are the average and standard deviation of three independent replicated experiments.

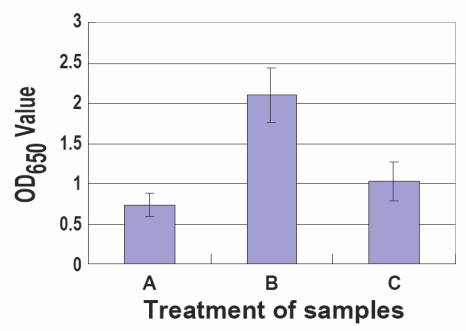


Figure 10. Golden proteins are active against PVY^{O} infection. OD_{650} values were measured on PVY^{O} infected cv. Winter Valley after the application of Golden protein. PVY^{O} infected and noninfected Winter valley was used as possitive control and negative control respectively. A : PVY^{O} non infected Winter valley, B : PVY^{O} infected Winter valley, C : PVY^{O} infected after the treatment of Golden valley protein in cv. Winter valley.

6. *Ry*$_{golden}$ gene construction strategy and sequence differences

Ry gene was cloned for sequencing analysis and development of anti-PVY^{O} potato. The two fragments of *Ry* gene left(1.3kb) and right(2.2kb) were amplified by using primers, Sense (RyF10) 5'-ATCCCGGGATGGCATCATCATCTTCTTCTTC-3', antisense (RyIR) 5'-AAGCATGCAATATCTAGAAATATCTCTTGA-3', Sense (RyIF) 5'-TTGCATGCTTCTTAAGAGGGAGAAAACAAA-3', antisense (RyR10) 5'-TACCCGGGCTCACATTGTCAACATCTTCAGT-3' respectively Both RT-PCR products were assembled on SphI linker site of cloning vector pUC19. Finally, the full length of *Ry* gene was digested and cloned on XmaI site of pBI121 binary vector. (Figure 11) Analysis of *Ry* gene sequence was showed that there were about 91% sequence homologs between *Solanum tuberosum subsp. andigena* and *Solanum tuberosum* **L.** *cv. Golden valley*. (Figure 12) Our second phase research work is going on to develop virus resistant potato cultivars by introducing, *Ry* gene and bio-pesticides for other legume crops simultaneously.

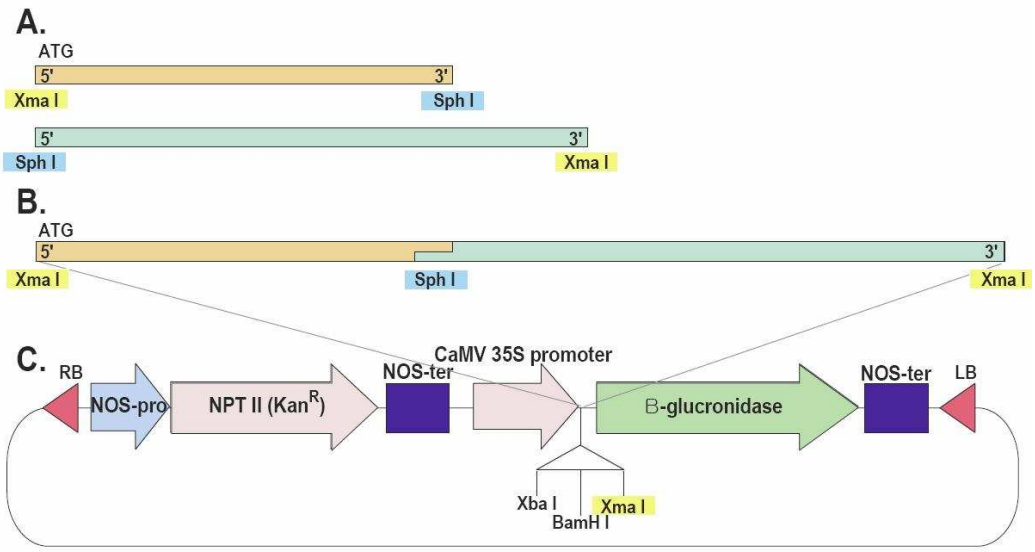
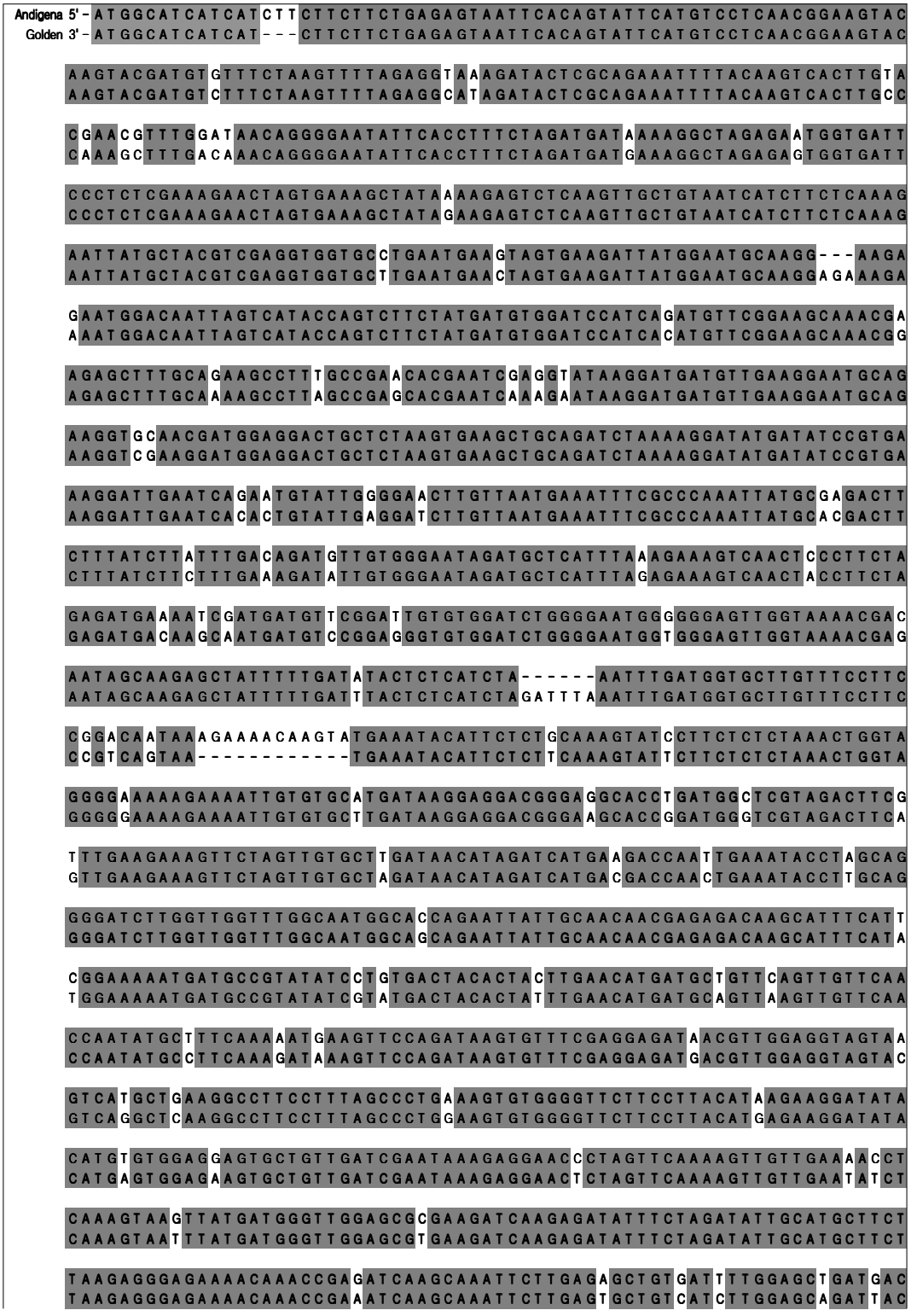


Figure 11. Strategy for construction. The two fragments of *Ry* gene left (1.3 kb) and Right (2.2 kb) were amplified by using primers, Sense (RyF10) 5'-AT**CCCGGG**ATGGCATCATCATCTTCTTCTTC-3', antisense (RyIR) 5'-AAGCATGCAATATCTAGAAATATCTCTTGA-3', Sense (RyIF) 5'-TTGCATGCTTCTTAAGAGGGAGAAAACAAA-3', antisense (RyR10) 5'-TA**CCCGGG**CTCACATTGTCAACATCTTCAGT-3' respectively Both RT-PCR products were assembled on SphI linker site of cloning vector pUC19. C: Finally the full length of *Ry* gene was digested and cloned on XmaI site of pBI121 binary vector.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Golden 3' - ATGGCATCATCAT---CTTCTTCTGAGAGTAATTCACAGTATTCATGTCCTCAACGGAAGTAC
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CGAACGTTTGGATAACAGGGGAATATTCACCTTTCTAGATGATAAAAGGCTAGAGAATGGTGATT
CAAAGCTTTGACAAACAGGGGAATATTCACCTTTCTAGATGATGAAAGGCTAGAGAGTGGTGATT
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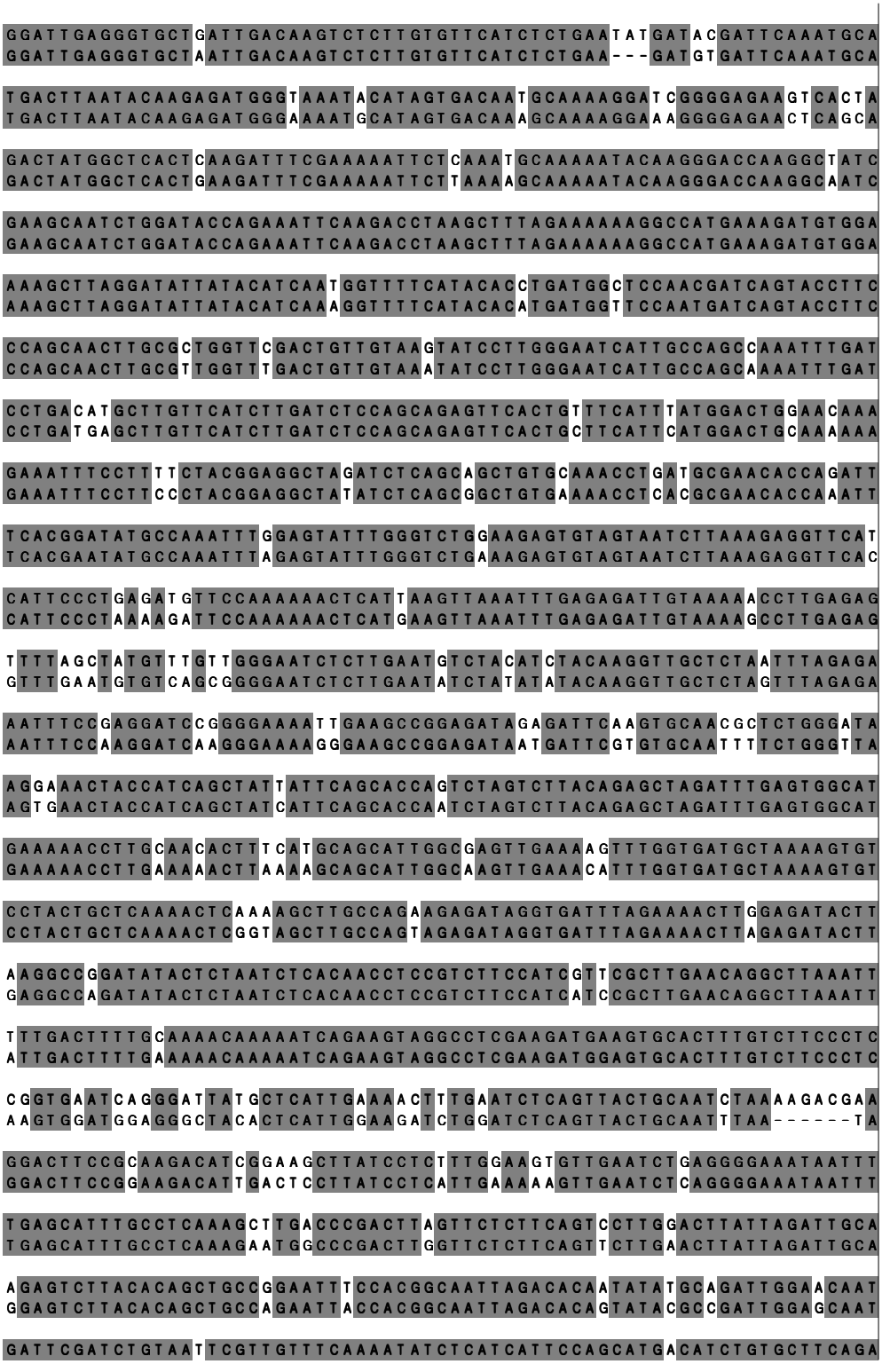

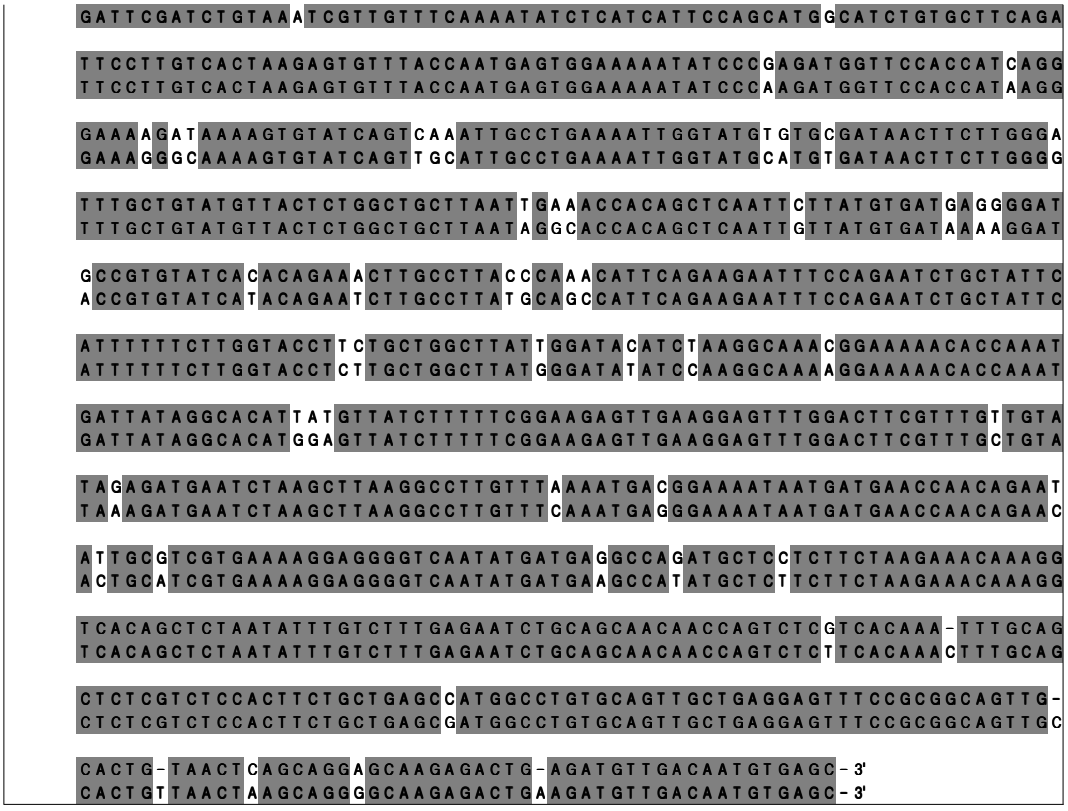

Figure 12. The difference of sequence between *Solanum tuberosum subsp. andigena*(upper lane) and *Solanum tuberosum* **L.** *cv. Golden valley*(lower lane) was length between 3,535 bp that was *Solanum tuberosum subsp. andigena* and 3,546 bp that was *Solanum tuberosum subsp. andigena.* there were 91% sequence homologs.

적 요

감자는 인류의 식량자원에 큰 몫을 차지하고 있는 중요한 작물이며, 식생활의 서구화로 인해 그 수요 또한 급증하고 있지만 바이러스 등에 대한 피해로 생산량에 큰 차질을 가져오고 있다. 현재 감자에서 피해가 가장 큰 것은 감자 잎말림 바이러스병, 감자바이러스 Y, 감자바이러스 X 등 3종류이며, 그 중 잎말림 바이러스병은 감자 바이러스병 중에서 피해가 가장 높으며 품종에 따라서는 85%이상의 감수를 가져온다. 현재 우리 나라에서는 감자 잎말림 바이러스병에 의한 피해는 감소되었으며, 감자 바이러스 Y에 의한 피해가 제일 큰 실정이다. 감자 바이러스 Y에 감염된 식물체는 2-3주가 지난후 모자이크 반점, 황화현상, 잎의 괴사, 잎맥의 투명화 등의 생태학적인 변화를 보이며 15~75%의 수확량 감소를 가져온다.

본 연구는 12종류의 감자중에서 PVY에 감염이 잘 되는 감자인 윈터밸리와 감염이 잘 되지 않는 감자인 골든밸리를 선별하였고, PVY에서 유래된 *CP*(Coat protein) gene의 발현양상에 따른 *Ry* gene의 발현양을 DAS-ELISA(면역화학적인 기법을 이용한 단백질의 분석방법)와 RT-PCR(역전사 중합효소 연쇄반응)을 통해 확인함으로써 감자 바이러스 Y와 *Ry* gene의 상관관계를 규명했다. 이 결과에 따라 감염이 잘 되지 않는 감자종인 골든밸리에서 단백질을 추출하고, 감염이 잘 되는 감자종인 윈터밸리에 에 처리한 후 DAS-ELISA를 통해 바이러스의 CP protein의 양을 측정한 결과 PVY^{O}에 저항성이 향상됨을 확인하였다. 이에 윈터밸리에 *Ry* gene을 overexpression하기 위해 식물발현 벡터인 pBI121에 construction을 하였으며, 염기서열을 분석함으로써 NCBI gene bank에 나온 *Solanum tuberosum subsp. andigena*의 염기서열과 비교한 결과 약 91%의 상동성을 보였다. 이에 우리는 종에 따라 *Ry* gene의 길이 및 염기서열이 서로 다름을 확인하였으며 그 염기서열의 차이에 따라 PVY에 대한 저항성이 결정됨을 확인하였다. 이 실험 결과는 *Ry* gene을 이용하여 바이러스 내성이 있는 새로운 감자종을 생산하는데 크게 기여할 것이다.

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감 사 의 글

이 논문이 완성되도록 도와주신 주위의 여러분들께 우선 감사를 드립니다. 학위과정중에 끊임없는 관심과 사랑으로 다년간 지도하여 주시고 이끌어 주신 정현숙 지도교수님께 진심으로 감사드리며, 학위과정 중 저에게 많은 가르침과 조언을 해주신 박 역 교수님, 양 영기 교수님, 김 성준 교수님, 이 정섭 교수님, 전 홍성 교수님께 감사드립니다. 실험에 필요한 peptide 및 동결건조를 흔쾌히 허락해주신 박윤경 교수님, 단백질소재연구센터의 함경수 소장님께 정말 감사 드립니다. 힘든 유학생활에도 실험에 대한 조언을 아끼지 않고 영문 수정에 이르기까지 많은 도움을 준 Giri Raj Tripathi, Dilli Prasad Paudyal과 실험방법 등을 가르쳐주신 박 재균 선배님께 정말 감사드립니다.

항상 옆에서 꾸짖어주시고 조언을 해주신 황 인덕 선생님, 어려고 힘든 시기가 다가올 때마다 항상 위로해주었던 박 가영, 저의 궂은 일을 옆에서 봐주고 도와주신 박 재영 선배님, 항상 옆에서 힘내라고 용기를 주었던 실험실원 정 주영, 항상 옆에 있다는 것으로만으로도 힘이 되는 나의 동기들인 조 홍석, 김 상용, 박 으뜸, 허 봉석, 김 진영, 실험에 필요한 peptide를 제공해주고, 동결건조 해주었던 김 미현,비록 지금은 옆에 없지만 항상 묵묵히 제 실험을 옆에서 물심양면으로 도와주었던 이 창수에게 정말 감사드립니다. 또한 백신감자를 제공해 주신"Potato valley"회사의 인 학태 교수님과 감자바이러스를 제공해 주신"Plant Virus Genbank"관계자분들께 고마움을 전합니다. 항상 끊임없는 사랑과 믿음으로 저에게 든든한 후원자 역할을 해주신 아버지, 어머니, 하나뿐인 제 동생에게 정말 감사 드립니다. 마지막으로 이 논문이 완성되기까지 후원해 주신 모든 분들께 다시 한 번 깊은 감사를 드립니다.

2007년 12월 1일

박 영 민

저작물 이용 허락서

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논문제목	한글 골든밸리감자에서 바이러스 저항성 유전자의 발현 및 분석				
	영문 Expression and Engineering of Virus Resistant Gene in *Solanum tuberosum* L. *cv. Golden valley*				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(0) 반대( )

2008년 2월 28일

저작자: 박 영 민 (인)

조선대학교 총장 귀하