Pak. J. Bot., 41(2): 945-954, 2009.

DEVELOPMENT OF MOLECULAR RESISTANCE IN POTATO AGAINST POTATO LEAF ROLL VIRUS AND POTATO VIRUS Y THROUGH *AGROBACTERIUM*-MEDIATED DOUBLE TRANSGENESIS

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Abstract

Potato leafroll virus (PLRV) and potato virus Y (PVY) are the two major viral problems for the potato production all over the world. Transgenic approaches involving the expression of viral genes are being developed to provide protection for plants against viral diseases. The purpose of this study was to develop double transgenic plants of potato using PLRV replicase and PVY coat protein genes tandemly placed in a single T-DNA transformant through *Agrobacterium*-mediated transformation. A total of 17 lines of putative transformants of potato cv. Desiree were generated from kanamycine resistant calli originated from co-inoculation of separate *Agrobacterium* cultures containing PVY CP and PLRV replicase genes. Shoots were excised and cultured onto shoot medium containing 250mg/L cefotaxime and 50mg/L kanamycin sulfate in test tubes. Polymerase chain reaction (PCR) analysis was conducted of 39 plants of 16 transformed lines using primers each of PVY CP and PLRV-replicase genes; 10 plants of 8 lines and 7 plants of 6 lines showed presence of of PVY CP and PLRV-replicase genes, respectively. However, 22 plants of 14 lines harbored both PVY-CP (508bp) and PLRV-replicase (449bp) genes. Sixteen plants of 11 double transgenic lines that showed high level of expression of both PLRV-replicase and PVY-CP genes. Transformants and control standards were exposed to field virus infection augmented by placement of aphids on *Datura* leaves infected with PLRV. Two clones (Des (CP+LR) 9.1 and Des (CP + LR) 9.2) showed incidence of infection statistically lower than the lowest infection of one of the standards (Ranger). The Monsanto clone (21-350) showed no infection. The two resistant clones may be identical as they were derived from the same callus.

Introduction

During the early 1980s, an alternative approach emerged from various laboratories to engineer and incorporate stable resistance in susceptible crops by introducing a genome segment of the pathogen into the plant genome (Hamilton, 1980; Sanford & Johnston, 1985). The theory of pathogen derived resistance (PDR) became a reality in 1987 when Powel-Abel *et al*., (1986) demonstrated that tobacco (*Nicotiana tabacum* cv. Xanthi) plants which had been stably transformed by *Agrobacterium tumefaciens* to express the coat protein (CP) gene of tobacco mosaic tobamovirus (TMV) were resistant to the virus. Between 1987 and 1995, large number of reports appeared on coat protein mediated resistance (CPMR), targeted against members of all the major groups of plant RNA viruses. These have been reviewed extensively (Beachy *et al*., 1990; Wilson, 1993; Lomonossoff, 1995; Beachy, 1997; Lecoq, 1997; Arif & Hassan, 2000). A second revolutionary approach appeared in 1990 when it was shown that transgenic plants expressing the TMV sequence encoding for non-structural, enzymatic protein, the RNA-

dependent RNA polymerase or replicase, had also conferred protection against high concentration of challenge inocula of TMV (100µg/ml) or TMV RNA (300µg/ml). The replicase gene mediated resistance (RGMR) operates only against very closely-related viruses, even more so than CPMR, but seemed to confer "immunity" by inhibiting all stages of the viral RNA replication cycle (Carr and Zaitlin, 1991; Carr *et al*., 1992). Several parallel systems were soon developed and studied, notably from pea early browning tobravirus (PEBV, MacFarlane & Davies, 1992), potato virus X potexvirus (PVX, Braun & Hemenway, 1992; Longstaff *et al*., 1993), cucumber mosaic cucumovirus (CMV, Anderson *et al*., 1992; Zaitlin *et al*., 1994; Carr *at al*., 1994), the most widely prevalent potato virus Y potyvirus (PVY, Audy *et al*., 1994), cyrnbidium ringspot tombusvirus (Rubino *et al*., 1993) and potato leafroll polerovirus (Thomas *et al*., 2000). A third revolution occurred in 1992 when Dougherty and colleagues (Lindbo & Dougherty, 1992a, b) first showed that a CP gene, mutated to lose its ability of making any protein at all *in planta*, could, in many transformed tobacco lines, confer very high levels of resistance (immunity) to the parent virus. Taken together the data on non-expressing lines or low expressing lines of transformed plants with replicase genes, Baulcombe (1994), Dougherty *et al*., (1994), Silva-Rosales *et al*., (1994), Smith *et al*., (1994), Prins & Goldbach, (1996), Palukaitis & Zaitlin (1997) independently developed theories on RNA-mediated resistance strategies and their operation through a RNA sequence specific, host cell cytoplasmic, RNA degradation pathway; the same pathway which degrades over-expressed, unnecessary, faulty or already-translated cellular mRNAs that causes the genetic phenomena known as co-suppression. From this discussion, it is evident that the significance of PDR for developing virus resistant plants is well established. Hopefully, in the near future, more cases of PDR against different viruses, using different and broad-spectrum chimeric gene cassettes from virus genome, in various crops, will be developed. The present study will serve as basic source of information to exploit multiple trangenes using *Agrobacterium* in co-inoculation techniques.

Materials and Methods

Source of potato germplasm and transgenes: Potato cultivar Desiree was obtained from NRSP-6, Sturgeon Bay, WI, as axenically grown plants in tissue culture and Norkotah from tissue culture facility at USDA, ARS, Prosser, WA, USA and transgene constructs were obtained from sources given below:

Identification	Viral cistron	Vector	Promoter	Source
RC4pBinPA-ubi3	untranslateable PVY coat protein	*Agrobacterium tumefasciens*	*ubi3*.	Bill Belknap, USDA/ARS, Albany, CA
RC435S	untranslatable PVY coat protein	*A. tumefaciens*.	*CaMV35S*	William Dougherty (Smith *et al*., 1995)
PLRV-Replicase	expressible replicase of PLRV	*A. tumefaciens*	*ubi3*	Bill Belknap, USDA/ARS., Albany, CA

Characterization of plasmid clones containing transgene constructs: Plasmid clones containing transgenes in *Agrobacterium* (RC4pBinPAubi3P, LBA4404pBinplus PLRV-Replicase, RC435S) were grown in 10ml of LB (10g bacto-tryptone, 5g yeast extract, 5g NaCl for 1 liter, pH 7.2) in 250 ml conical flasks at 28°C for 16-24h with continuous shaking at 250 rpm. A small amount (2-5µl) of overnight culture was spread on LB agar

(10g bacto tryptone, 5g yeast extract, 5g NaCl, 15g bacto agar, per liter, pH 7.2) containing 50 mg/L kanamycin and Petri plates were incubated at 28°C for 24-48h. Single colonies were inoculated separately in 10 ml of LB medium containing 50mg/L kanamycin for 24 h at 28°C with shaking at 250 rpm.

Agrobacterium plasmid mini-preps were made using GeneluteTM (GeneElute Miniprep binding column, Sigma) plasmid mini prep kit (Sigma). One to 5 ml of an overnight recombinant *Agrobacterium* culture was centrifuged at 12000g for 5 min., and re-suspended in 200µl of re-suspension solution. Bacterial cells were lysed by adding 200 µl lysis solution. The content of the tube was mixed by gentle inversion (6-8 times) until the mixture became clear and viscous. The cells were precipitated by adding 350 µl of the neutralization/ binding solution and mixed with gentle inversion of tubes 4-6 times. The solution was pelleted at 12,000g for 10 min. The clear lysate was washed through a wash column with 750µl diluted wash solution to the column, and centrifuged at 12,000g for 30 sec., to 1 min. The DNA was eluted into a fresh tube by adding 100µl elution solution and concentrated at 12,000g for 10 min. The recovery and purity of DNA was determined by spectrophotometeric analysis. The ratio of absorbance was in between 1.7 to 1.9. The size and quality of DNA was determined by agarose gel electrophoresis.

The identity of *Agrobacterium* was confirmed using alpha-keto lactose test. A single *Agrobacterium* colony was streaked on lactose agar and incubated at 28°C for 16-20h. Overnight bacterial culture was flooded with Benedict's solution (add Sol. II to Sol. I, make the volume up to 100ml with sterile distilled water). [Sol. I: 17.3g Sodium citrate, 10g Na2CO3 in 80ml sterile distilled water, heat to get into solution; filter through a Whatman 1 filter paper, bring to 85 ml volume with sterile distilled water; Sol. II 1.73g CuSO4.5H2O in 10 ml of distilled water, heat to get into solution]. The boundaries of the *Agrobacterium* culture turned yellow after 10 min to an hour.

Confirmation of coat protein and replicase genes in plasmid using polymerase chain reaction amplification: Polymerase chain reaction (PCR) amplification was used to confirm the presence of gene inserts in *Agrobacterium*. *Agrobacterium* plasmid minipreps was made using Genelute plasmid minipreps kit (Sigma). PCR amplification was performed in PTC-200 Thermal Cycler (MJ Research, Watertown, MA, USA) for 94°C for 2 min., 94°C for 15 sec, 58°C for 2 min., and 72°C for 10 min., (total 30 cycles). Primers for the PVY-CP (based on the Idaho PVY sequence) and PLRV-replicase genes were obtained from Genosys Biotechnologies (The Woodland, Texas) and have the following sequences:

PVY-CP (YID124): 5′-CGTACTGTGCCGAGAATCAA-3′
PVY-CP (YID631): 5′-ACTGGTGTTCGTGATGTGTGAAC-3′
PLRV-replicase (lrar2033): 5′-AGACTCTGCCAAGTTCATCC-3′
PLRV-replicase (lrar2481): 5′-AGCTCTTCTTGCGCTCATATC-3′

The PCR reaction mixture (50µl) contained 20 pmol of downstream and upstream primers, 5µl 200µM solution of each of four deoxynucleotide triphosphates (dNTPS), 5µl 10xPCR buffer (10mM tris-HCl, pH 8.4 containing 50mM KCl and 1.5mM MgCl2), 5µl Rediload, 0.2µl Taq DNA polymerase (Promega) and 28µl sterile water and 0.2-0.5µl of DNA template. The samples were overlaid with light parafilm oil (50µl) and subjected to 30 cycles of heating and cooling in a Thermal Cycler. Amplified products were electrophoresed in 1% agarose gels in tris-EDTA buffer containing 0.5µg/ml ethidium bromide and 100bp molecular size marker (Promega). The specific bands were visualized under UV light using Quantity One ® 4.1 for Windows (Bio-Rad).

Plant micropropagation: Plant material (internodes and leaf discs) for transformation of potato cultivar Desiree and Norkotah was grown axenically on Murashige & Skoog medium (Murashige & Skoog, 1962) shoot medium (4.30g MS salts (Sigma Chemical Co., St. Louis), 37.0µm glycine, 4.0µM nicotinic acid, 2.0µM pyridoxine monohydrochloride, 1.0 µM thiamine hydrochloride, 0.6µM folic acid, 0.2µM d-biotin, 100mg myo-Insitol, 20µg napthaleneacetic acid, 25-30g sucrose and 8.0 g agar per liter, pH 5.6) in test tubes and under 12 h fluorescent light (1-1.4µ E Sec^{-1} m^{-2}).

Plant transformation with co-inoculation of PVY-CP and PLRV-replicase genes: The RC4 CP and PLRV-replicase genes were introduced into two potato cultivars by co-inoculation techniques. Method used for transformation was essentially the same as described by Arif *et al*., (2009). *Agrobacterium* culture containing RC4 CP and PLRV-replicase were grown 16-20 h in LB with 50mg/L kanamycin sulfate at 28˚C with continuous shaking at 250rpm. *Agrobacterium* cultures were grown to an OD_{600} 0.6-0.7, centrifuged at 7,000 rpm for 10 min., at 20ºC. The pellet was washed twice with MS liquid medium. The final pellet was re-suspended with an equal volume of MS liquid medium. Internodal sections of 5-8 mm length were cut from the stem of 3-5 wk-old axenically grown Desiree and Norkotah plants. The internodes were soaked in MS liquid medium until *Agrobacterium* culture inoculum was ready. The internodes were inoculated with *Agrobacterium* culture containing RC4 CP for 15-20 min in Petri plates sealed with parafilm at RT with gentle shaking on a bench top shaker. The *Agrobacterium* culture was drained off from Petri plates, and internodes were quickly blot dried on sterile Whatman filter paper, and placed into *Agrobacterium* culture containing PLRV-replicate gene. The plates were sealed with parafilm and incubated for 15-20 min with gentle shaking on bench top shaker at RT. The internodes were blot-dried on sterile Whatman filter paper and placed on CIM and incubated at 21˚C in culture room for 2-3 days with restricted light. The stem pieces were transferred to 3C5ZR containing 50mg/L kanamycin sulfate and 250mg/L cefotaxime. The internodes (explants) were transferred to fresh medium at weekly intervals. The callus formation was commenced during 2-3 week and shoots were harvested between 8-12 wk. The shoots were excised and grown on MS shoot medium containing 50mg/L kanamycin sulfate and 250mg/L cefotaxime in tissue culture test tubes. All *In vitro* culture was at 21˚C under continuous light provided by Gro-Lux fluorescent bulbs.

PCR analysis of putative transformants with PVY-CP and PLRV-replicase genes: Potato genomic DNA was isolated as described by Arif *et al*., (2009). PCR amplification was performed in Thermal Cycler. Two separate PCR mater mixtures were prepared using 5µl 10 x Taq polymerase buffer (plus MgCl2) (Promega), 5ul Rediload, 5ul 200µm dNTPS solution, 1.0 µl each of reverse and forward primers (20pmol/µl) (for RC4 analysis YID124 and YID124 used as reverse and forward primers, respectively, whereas for replicate gene lrar 2033 used as reverse and lrar2481 as forward primer), 28 µl of sterile distilled water and 0.2 µl Taq DNA polymerase (Promega). The contents were thoroughly mixed and 45 µl of mater mixture was separately dispensed to each of 0.5 ml PCR tubes for RC4 and replicase gene analysis. Five microliters each of genomic DNA from RC4 CP and PLRV-replicase gene transformants was added into respective tube, approximately 2 drops of mineral oil was placed on each tube. The PCR reaction was run on PTC-200 Thermal Cycler (MJ Research, Watertown, MA). PCR amplification was performed for 30 cycles and PCR products were analyzed as reported by Arif *et al*., (2009).

Table 1. Final selection of double transgenic plants of potato cv. Desiree transformed with PVY coat protein and PLRV replicase genes.

Transgenic line	Plant identification	Gene presence[1]	
		RC4CP (508bp)	PLRV-rep. (449bp)
D-RC4(CP)+LR(R)-1	1.1	+++	+++
D-RC4(CP)+LR(R)-6	6.2	+++	+++
D-RC4(CP)+LR(R)-6	6.3	++	+++
D-RC4(CP)+LR(R)-7	7.1	++	+++
D-RC4(CP)+LR(R)-7	7.2	+++	+++
D-RC4(CP)+LR(R)-9	9.1	++	+++
D-RC4(CP)+LR(R)-9	9.2	+++	+++
D-RC4(CP)+LR(R)-10	10.1	+++	+++
D-RC4(CP)+LR(R)-11	11.2	++	+++
D-RC4(CP)+LR(R)-12	12.2	++	+++
D-RC4(CP)+LR(R)-13	13.1	+++	+++
D-RC4(CP)+LR(R)-13	13.3	+++	+++
D-RC4(CP)+LR(R)-14	14.1	+++	+++
D-RC4(CP)+LR(R)-15	15.1	++	+++
D-RC4(CP)+LR(R)-15	15.2	+++	++
D-RC4(CP)+LR(R)-16	16.1	++	+++
D-RC4(CP)+LR(R)-16	16.2	+++	++

[1]Transformant selection was made on the basis of polymerase chain reaction (PCR) analysis using a set of primers to amplify coat protein gene of PVY and replicase gene of PLRV. The amplified products were electrophoresed in 1% agarose gels in tris-EDTA buffer containing 0.5µg/ml ethidium bromide. The specific bands were visualized under UV light using Quantity One ® 4.1 for Windows (Bio-Rad). The specific bands of PVY CP gene (508bp) and PLRV-(449bp) were assessed as: + = weak reaction, ++ = medium reaction and ++++ = strong reaction.

Field exposure to PLRV: Selected clones were propagated *In vitro* and transplanted in the field in the summer of 2004 at the Irrigated Agriculture Research and Extension Center, Prosser, WA. The proprietary Monsanto clone, 21-350, was propagated *In vitro* with PLRV replicase was kindly provided, propagated *In vitro* and transplanted, serving as a resistant standard. Green peach aphids (*Myzus persicae* Sulz.) were reared on PLRV infected *Datura tatula* plants. Six weeks after transplanting portions of *D. tatula* leaves with at least 20 apterous aphids were manually placed on the top of each plant. At harvest tubers were collected and two tubers each were sprouted in the greenhouse and grown to a height of 10 cm. Three samples were taken from each plant at apical, medial and basal positions on the plant and tested for PLRV using a two step ELISA test (Kaniewski & Thomas, 1988). Samples that reacted with an absorbance greater than 0.10 (at lambda = 450 nm) were classified as infected.

Results and Discussion

Production of double transgenic plants with potato virus Y coat protein and leafroll virus replicase genes: A total of 17 lines of putative transformants were generated from kanamycine resistant calli originated from co-inoculation of separate *Agrobacterium* cultures containing PVY CP and PLRV replicase genes (Fig. 1). Shoots were excised and cultured onto shoot medium containing 250mg/L cefotaxime and 50mg/L kanamycin sulfate in test tubes, for multiplication.

Polymerase chain reaction (PCR) analysis was conducted of 39 transformants of 16 transformed lines using primers set each of PVY CP and PLRV-replicase genes; 10 transformants of 8 lines and 7 plants of 6 lines showed expression of PVY CP and PLRV-replicase genes, respectively (Table 1; Fig. 2). However, 22 transformants of 14 lines

expressed both PVY-CP (508bp) and PLRV-replicase (449bp) genes (Table 1; Figs. 2 & 3). Among 22 transformants of 14 lines, 16 transformants of 11 double transgenic lines showed high level of expression of both PLRV-replicase and PVY-CP genes and these were selected for further propagation and evaluation of resistance against PLRV and PVY (Table 1; Fig. 3).

Transformants and control standards were exposed to field virus infection augmented by placement of aphids on *Datura* leaves infected with PLRV. Two clones (Des (CP+LR) 9.1 and Des (CP + LR) 9.2) showed incidence of infection statistically lower than the lowest infection of one of the standards (Ranger). The Monsanto clone (21-350) showed no infection. The two resistant clones may be identical as they were derived from the same callus (Fig. 4).

In recombinant technology, a number of strategies are in the process of development for the production of virus resistant plants. Some strategies provide high level of resistance in transformed plants in some situations but not all. If we considered PLRV a test case, CP mediated protection gives some resistance in Russet Burbank potatoes but still allows for the storage disease of net necrosis (Palukaitis & Zaitlin, 1997). On the other hand, replicase mediated protection gives resistance but no net necrosis. By comparison, CP mediated protection provides very adequate control of many diseases of annual plants (Beachy *et al*., 1990). This discussion leads us to think for the approaches for the transformation of plants using more than one type of gene for resistance to broaden the spectrum of resistance to other viruses and strains. The present study is the classical example of toward the development of broad spectrum resistance in potato against two major virus problem in worldwide (PVY and PLRV) by incorporating CP gene of PVY and replicase gene of PLRV in single transformant through *Agrobacterium*-mediated transformation of potato cv. Desiree. This is also a unique approach to incorporate genes of two viruses in single transformant through *Agrobacterium* co-inoculation technique.

Incorporation of two similar sequences (two CP genes or two replicase genes) would lead to recombination of such sequences and there is a possibility for the development of new viruses or virus strains or alteration in virus properties. This risk possibility was also overcome in present study by incorporating two dis-similar sequences (two different genes-CP and replicase) of different viruses to develop a broad spectrum resistance avoiding any risk of recombination and regeneration of "super virus". A part of the following question also addressed through present study of double transgensis: Does *trans*-encapsidation represent a significant risk? Trans-encapsidation, if occurs, will not lead to a permanently expanded host range of trans-encapsidated RNA. Trans-capsidation is known to occur in mixed virus infection because many plants are infected simultaneously by multiple viruses (Bendahmane & Beachy, 1999). It is possible to reduce the potential for trans-capsidation by using CP that is incapable of assembly or insect transmission yet is still capable of conferring CP-mediated resistance (Bendahmane & Beachy, 1999). It is important to gain more understanding of molecular, cellular, and structural mechanism of virus assembly, disassembly, replication and movement in order to be able to increase the efficacy of PDR and reduce or eliminate the biosafety concerns related to the use of PDR in agriculture. So far, the most common type of PDR in advance stages of development in CP-mediated resistance and government regulatory agencies of many countries approved the use of CP genes in agriculture. It is anticipated that other types of PDR will be approved in the near future. The present study of double transgensis in potato by combining CP gene of PVY and replicase gene of PLRV in single transformant will serve as base line for future development of broad spectrum PDRs.

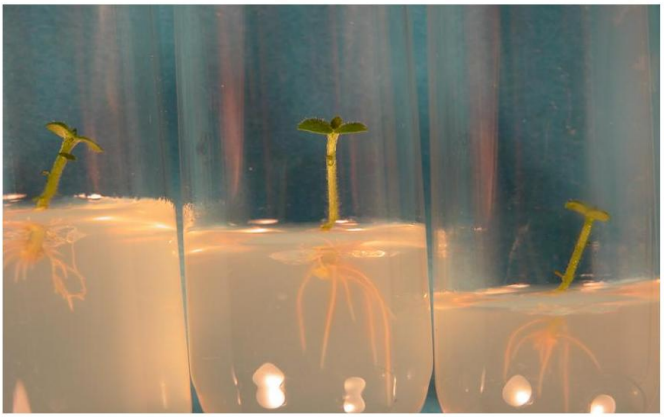

Fig. 1. Double transgenic plants of potato cv. Desiree 14 wk after *Agrobacterium tumefaciens*-mediated transformation.

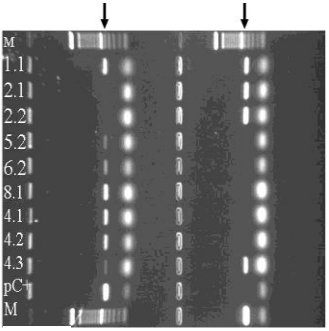

Fig. 2A

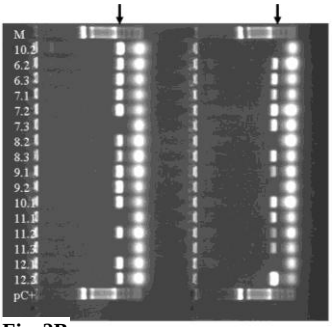

Fig. 2B

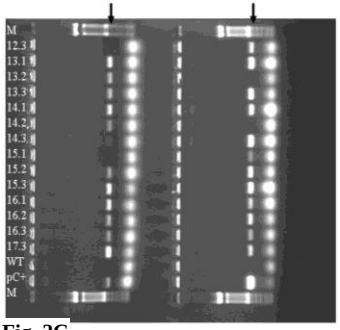

Fig. 2C

Fig. 2. Polymerase chain reaction (PCR) analysis of potato cv. Desiree transformed with PVY coat protein and PLRV-replicase genes in single transformant. Amplified products were electrophoresed in 1% agarose gels in tris-EDTA buffer containing 0.5µg/ml ethidium bromide. The specific bands were visualized under UV light using Quantity One ® 4.1 for Windows (Bio-Rad). Arrows indicating the the specific bands of PVY-CP and PLRV-replicase genes. PCR product size of 508bp for PVY-CP and 449bp for the PLRV-replicase gene. M is DNA marker of 100bp (Promega). Lanes 1.1 to 17.3 (Fig 2A-C) are the transformed lines containing both PVY-coat protein and PLRV-replicase genes in single transformant; WT is wild-type (non-tranformed) and pC+ was the purified DNA from *Agrobacterium*-plasmid clones containing PLRV replicase (left) and PVY-CP (right) genes separately, used as positive control in PCR reaction.

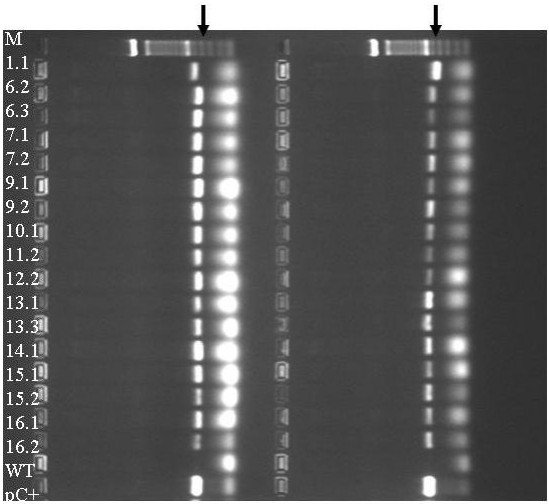

Fig. 3. Polymerase chain reaction (PCR) analysis of potato cv. Desiree transformed with PVY coat protein and PLRV-replicase genes in single transformant. Amplified products were electrophoresed in 1% agarose gels in tris-EDTA buffer containing 0.5µg/ml ethidium bromide. The specific bands were visualized under UV light using Quantity One ® 4.1 for Windows (Bio-Rad). Arrows indicating the the specific bands of PVY-CP and PLRV-replicase genes required PCR product size of 508bp for PVY-CP and 449bp for the PLRV-replicase gene. M is DNA marker of 100bp (Promega). Lanes 1.1 to 16.2 are the selected transformed lines containing both PVY-coat protein and PLRV-replicase genes in single transformant; WT is wild-type (non-tranformed) and pC+ was the purified DNA from *Agrobacterium*-plasmid clones containing PLRV replicase (left) and PVY-CP (right) genes separately, used as positive control in PCR reaction.

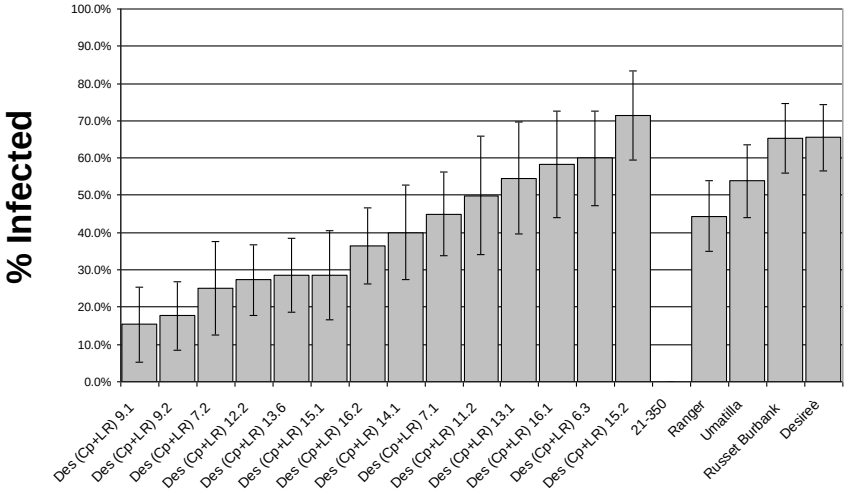

Fig. 4. Percent infection by PLRV in winter indexing of daughter tubers using ELISA. Standard errors were calculated using binomial standard error (p-standard error [(9p(1-p))/ (N = sample size)]$^{1/2}$.

Acknowledgements

We wish to express our gratitude and appreciation to Ching-Pa Yang, USDA/ARS, for technical assistance and supply of materials pertinent to this work. The financial support of Fulbright Scholar Program, U.S. Department of the State (USDS), Bureau of Educational and Cultural Affairs, Council for International Exchange of Scholars (CIES) is gratefully acknowledged.

References

Anderson, J.M., P. Palukaitis and M. Zaitlin. 1992. A defective replicase gene induces resistance to cucumber mosaic virus in transgenic tobacco plants. *Proc. Nat. Acad. Sci*., *USA*, 89: 8759-8763.

Arif, M. and S. Hassan. 2000. Pathogen-derived resistance against plant viruses: postscript and prospects. *Pak. J. Biol. Sci*., 3: 1-9.

Arif, M., P.E. Thomas, J.M. Crosslin and C.R. Brown. 2009. *Agrobacterium*-mediated transformation of potato cv. Desiree using potato leafroll virus replicase and potato virus Y coat protein genes and development of replicase mediated resistance against natural infection of potato leafroll virus. *Pak. J. Bot*., 41: (In press).

Audy, P., P. Palukaitis, S.A. Slack and M. Zaitlin. 1994. Replicase mediated resistance to potato virus Y in transgenic tobacco plants. *Mol. Plant-Microbe Interact*., 7: 15-22.

Baulcombe, D.C. 1994. Replicase-mediated resistance: a novel type of virus resistance in transgenic plants. *Tren. Microbiol*., 2: 60-63.

Beachy, R.N. 1997. Mechanism and application of pathogen derived resistance in transgenic plants. *Curr. Opin. Biotech*., 28: 451-474.

Beachy, R.N., S. Loesch-Fries and N.E. Tumer. 1990. Coat protein mediated resistance against virus infection. *Ann. Rev. Phytopathol*., 28: 451-474.

Bendahmane, M. and R.N. Beachy. 1999. Control of tobamovirus infections *via* pathogen-derived resistance. *Adv. Virus Res*., 53: 369-386.

Braun, C.J. and C.L. Hemenway. 1992. Expression of amino acid portions or full-length genes in transgenic plants confers resistance to potato virus X. *Pl. Cell*, 4: 735-744.

Carr, J.P. and M. Zaitlin. 1991. Resistance in transgenic plants expressing a nonstructional gene sequence of tobacco mosaic virus is a consequence or markedly reduced virus replication. *Mol. Plant-Microbe Interact*., 4: 579-585.

Carr, J.P., A. Gal-On, P. Palukaitis and M. Zaitlin. 1994. Replicase mediated resistance to cucumber mosaic virus in transgenic plants involves suppression of both virus replication in the inoculated leaves and long-distance movement. *Virology*, 199: 439-447.

Carr, J.P., L.E. Marsh, G.P. Lomonossoff, M.E. Sekiya and M. Zaitlin. 1992. Resistance to tobacco mosaic virus induced by the 54-kDa gene sequence require expression of the 54-kDa protein. *Mol. Plant-Microbe Interact*., 5: 397-404.

Dougherty, W.G., J.A. Lindbo, H.A. Smith, T.D. Parks and W.M. Proebsting. 1994. RNA-mediated virus resistance in transgenic plants: exploitation of a cellular pathway possibly involved in RNA degradation. *Mol. Plant-Microbe Interact*., 7: 544-552.

Hamilton, R.I. 1980. Defense triggered by previous invaders: viruses. In: *Plant Disease: An Advance Treatise*. (Eds.): J.G. Horsfall, E. and B. Cowling, Vol. 5 pp. 279-303. Academic Press, New York.

Kaniewski, W. and P.E. Thomas. 1988. A two-step ELISA for rapid, reliable detection of potato viruses. *Am. Potato J*., 65: 561-571.

Lecoq, H. 1997. Molecular biology and genetic engineering: new prospects for control of viral diseases of plants. *Comptes Rendus de I'Academie d'Agriculture de France*, 83: 153-160.

Lindbo, J.A. and W.G. Dougherty. 1992a. Pathogen-derived resistance to a potyvirus: immune and resistance phenotypes in transgenic tobacco expressing altered from a potyvirus coat protein nucleotide sequence. *Mol. Plant-Microbe Interact*., 5: 144-153.

Lindbo, J.A. and W.G. Dougherty. 1992b. Untranslateable transcripts of tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. *Virology*, 189: 725-733.

Lomonossoff, G. P. 1995. Pathogen-derived resistance to plant viruses. *Ann. Rev. Phytopathol*., 33: 323-343.

Longstaff, M., G. Brigneti, F. Boccard, S. Chapman and D. Baulcombe. 1993. Extreme resistance to potato virus X infection in plants expressing a modified component of putative viral replicase. *EMBO Journal*, 12: 379-386.

MacFarlance, S.A. and J.W. Davies. 1992. Plant transformed with a region of the 201-kilodalton replicase gene from pea early browning virus RNA 1 are resistant to virus infection. *Proc. Natl. Acad. Sci. USA*, 89: 5829-5833.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant*., 15: 473-497.

Palukaitis, P. and M. Zaitlin. 1997. Replicase-mediated resistance to plant virus disease. *Adv. Virus Res*., 48: 349-377.

Powell-Abel P., R.S. Nelson, B. De, N. Hoffmann, S.G. Rogers, R.T. Fraley and R. Beachy. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science*, 232: 738-743.

Prins, M. and R. Goldbach. 1996. RNA-mediated resistance in transgenic plants. *Arch. Virol*., 141: 2259-2276.

Rubino, L., R. Lupo and M. Russo. 1993. Resistance to cymbidium ring spot tombus virus infection in transgenic *Nicotiana benthamiana* plants expressing a full-length viral replicase gene. *Mol. Plant-Microbe Interact*., 6: 395-405.

Sanford, J.C. and S.A. Jhonston. 1985. The concept of parasite-derived resistance genes from parasites own genome. *J. Theo. Biol*., 113: 395-405.

Silva-Rosales, L., J.A. Lindbo and W.G. Dougherty. 1994. Analysis of transgenic tobacco plants expressing a truncated from of a potyvirus coat protein nucleotide sequence. *Mol. Plant-Microbe Interact*., 24: 929-939.

Smith, H.A., S.L. Swaney, D.T. Parks, E.A. Wernman and W.G. Dougherty. 1994. Transgenic plant virus resistance mediated by untranslated sense RNAs: expression, regulation, and fate of nonessential RNAs. *Pl. Cell*, 6: 1441-1453.

Thomas, P.E., E.C. Lawson, J.C. Zalewski, G.L. Reed and W.K. Kaniewski. 2000. Extreme resistance to potato leafroll virus in potato cv. Russet Burbank mediated by the viral replicase gene. *Virus Res*., 71: 49-62.

Wilson, T.M.A. 1993. Strategies to protect crop plants against viruses: pathogen-derived resistance blossoms. *Proc. Natl. Acad. Sci. USA*., 90: 3134-3141.

Zaitlin, M., J.M. Anderson, K.L. Perry, L. Zhang and P. Palukaitis. 1994. Specificity of replicase-mediated resistance to cucumber mosaic virus. *Virology*, 201: 220-205.

(Received for publication 25 July 2007)