Progress in Biological Sciences
Vol. 5, Number 1, Winter / Spring 2015/19-31

Transient expression of coding and non-coding regions of PVY confer resistance to virus infection

Received: September 1, 2014; Accepted: November 1, 2014

Marzie Ghanbari Jahromi*[1] , Hassan Rahnama[2], Amir Mousavi [3] , Mohammad Reza Safarnejad[4], Sepide Kalatejari[1], Saeed Soheilivand[2]

1. Department of Horticultural Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran
2. Department of Tissue Culture and Gene Transformation, Agricultural Biotechnology Research Institute of Iran, Karaj, Iran
3. Department of Agricultural Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran
4. Department of Plant Viruses, Iranian Research Institute of Plant Protection, Tehran, Iran

ABSTRACT

One of the most efficient mechanisms by which plants protect themselves from invading viruses is the specific RNA-dependent silencing pathway termed post-transcriptional gene silencing (PTGS). In this mechanism, resistance to a virus is engineered through the expression of a segment of the virus genome in transgenic plants. Potato VirusY (PVY) is one of the most damaging viruses of potato, infecting most cultivars and causing significant yield losses throughout the world. The present study was performed to compare the efficiency of three construct containing different regions of 3′UTR (UR) and coat protein (CP) against PVY infection. Expression of homologous hairpin RNA to PVY in potato plant was carried out by transient gene expression of constructs with agro-infiltration followed by mechanical viral infection. Results showed that successful production of siRNAs confer resistance to two PVY strain. Comparison between transiently expressed constructs indicated that applying CP+UR PVY hairpin RNA was the most efficient RNAi construct to confer resistance. Resistance was found to have taken the form of immunity, since no viral particle could be detected in the upper leaves as shown by ELISA assay and Northern hybridizations. To the best of our knowledge, this is the first report on the application of 3'non-coding region of PVY in conferring complete resistance against virus in potato.

Keywords: potato, PVY, RNA silencing, transient expression.

* **Corresponding author:** hrahnama@abrii.ac.ir

Introduction

Pathogen-derived resistance (PDR) is a phenomenon whereby transgenic plants containing full-length or truncated gene sequences of a parasite are protected against damaging effects of the cognate or related pathogens (26). The application of PDR to plant viruses was discovered for the first time in transgenic tobacco (23) plant expressing the coat protein (CP) gene of TMV (*Tobacco mosaic virus*) having shown resistance or delayed infection by TMV. The successful application of PDR in controlling plant viruses has been firmly reported indifferent studies (1, 14, 16, 29, 32, 35). However, over the past decade, most examples of PDR for plant viruses are RNA-mediated (1, 14, 16, 30, 33, 35) and occur through the mechanism of post-transcriptional gene silencing (PTGS), which is also now commonly referred to as the antiviral pathways of RNA silencing. Several studies have shown that inverted repeat constructs encoding self-complementary RNAs (hairpin RNAs) from different parts of viral genes (CP, RNA-dependent RNA polymerase [RdRp], proteinase, movement proteins, satellite RNA, defective interfering RNA, and non-coding regions) can effectively induce RNA silencing and lead to high resistance frequencies in transgenic plants (3, 5,28).

The artificially introduced dsRNA mimics the presence of a double-stranded form of RNA produced during virus replication, triggering the initiation step of PTGS. This may lead to the production of small 21 to 24 nucleotide interfering RNAs (siRNAs), which are incorporated into the RNA-induced silencing complex (RISC) for the degradation of the cognate viral RNA (2, 8). Thus, the invading virus containing sequences homologous to the dsRNA is recognized and degraded by the plant's defense mechanism.

Potato virus Y (PVY), a member of the genus *Potyvirus*, is one of the most damaging viruses of potato, infecting most cultivars and causing significant yield and economical losses. It is a single-stranded RNA virus, which replicates via double-stranded (ds) RNA intermediates. Different parts of the *Potyvirus* genome have been used to produce virus-resistant plants by RNAi constructs: coat protein (18, 19), NIa protease (27), P1 (4, 17) and Nib (6) sequences (Fig. 1). Meanwhile, there is no report about using non-coding regions to produce RNAi constructs. Strong resistance was demonstrated to three different PVY strains by transgenic potatoes bearing a coatprotein-hpRNA construct (19). However, resistance was not always very strong (11,13), often strain-specific (17) and protection appeared almost always in only a few of the transgenic lines generated..

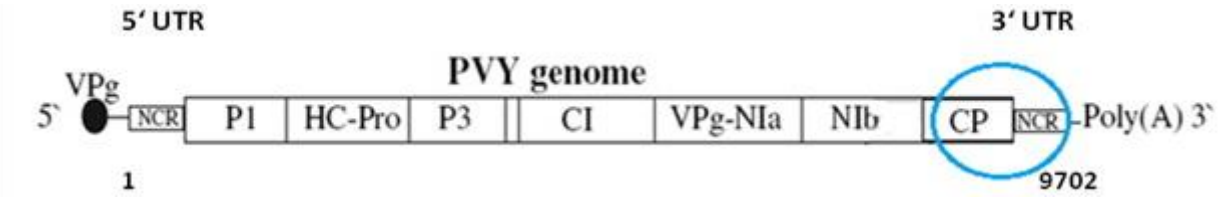

Figure 1. The genome structure and the functions of the proteins produced from the single-stranded messenger polarity RNA genome of a *potyvirus* (9.7kb). The genome contains short non-coding regions (NCR) flanking the single open reading frame, and a poly(A) tail at the 3'-end. P1: P1 protein, HC-Pro: Helper componentproteinase, P3: The third protein, CI: Cylindrical inclusion protein, NIa: Nuclear inclusion protein a, VPg: Viral genome-linked protein, NIb: Nuclear inclusion protein b, CP: Coat protein (17). CP and NCR (3' UTR) were selected to design primers.

Agrobacterium-mediated transient gene expression technique in plant leaves is a rapid and useful method for evaluating gene expression. Moreover, this expression system is often used for the identification of new viral suppressors of RNA silencing and for functional analysis of unidentified genes (23,30)

In this study, we compared the efficiency of three RNAi constructs from different segments of PVY RNA (CP, UR and CP+UR) for resistance to PVY. They were used for transient expression of homologous dsRNA to PVY in potato. The main objective of this study is to compare coding (CP) and non-coding region (3'UTR) to produce high resistance to PVY in potato plant.

Materials and Methods

RNAi vector construction

To design specific primers, conserved regions of PVY genome from coat protein (CP) and non-coding region (3'UTR) were initially selected (Fig. 1). cDNA was generated from total RNA of PVY infected tobacco plants by M-MuLV reverse transcriptase (Roche, Mannheim, Germany) and oligo $(dT)_{15}$ primer. Three fragments (320bp) from different regions of the 3′-untranslated region and coat protein of PVY were obtained from cDNA by specific primers (Table 1) and cloned into pGEM-T Easy vector. The restriction enzyme sites, *Nco*I and *Asc*I were considered in sense fragments while *Bam*HI and *Spe*I in antisense fragment. The PVY RNA iconstructs were assigned as CP (from coat protein region), UR (from 3′-untranslated region) and CP+UR (a segment of coat protein and 3′untranslated region). The ligation products were introduced into *E. coli* (DH5α). After confirmation of clones by PCR, plasmids were extracted and purified for sequencing. The correct trans formants containing PVY fragments were cloned in the sense and antisense orientation in the RNAi destination vector, pGSA1252.The recombinant constructs were then digested with *Pst*I and subcloned in pCAMBIA3300 harboring *bar* selectable marker. The recombinant vectors (Fig. 2) were introduced into *Agrobacterium tumefaciens* strain AGL1 by freeze and thaw method (24).

Table 1. Characteristics of specific primers for RNAi constructs

Primer name	Type orientation	Amplification region		Sequence
CP.se	Sense	Coat protein	F	5'- CCATGGAGTCAAACCCGAACAAAGGAAAAG -3'
			R	5'- GGCGCGCCAATGCACCAAACCATAAGCCCATG -3'
CP.anti	Antisense	Coat protein	F	5'- ACTAGTAGTCAAACCCGAACAAAGGAAAAG -3'
			R	5'- GGATCCAATGCACCAAACCATAAGCCCATG -3'
UR.se	Sense	3'-UTR	F	5'- CCATGGTAGTGTCTCTCCGGACGATATATAAG -3'
			R	5'- GGCGCGCCAATCACAACACCACCCAAGCAG -3'
UR anti	Antisense	3'-UTR	F	5'- ACTAGTTAGTGTCTCTCCGGACGATATATAAG -3'
			R	5'- GGATCCAATCACAACACCACCCAAGCAG -3'
CP+UR se	Sense	Coat protein & 3'-UTR	F	5'- CCATGGTTCAAATGAAGGCCGCAGCAT -3'
			R	5'- GGCGCGCCAATTAAAAGATAGAGTCACCAC -3'
CP+UR anti	Antisense	Coat protein & 3'-UTR	F	5'- ACTAGTTTCAAATGAAGGCCGCAGCAT -3'
			R	5'- GGATCCAATTAAAAGATAGAGTCACCAC -3'

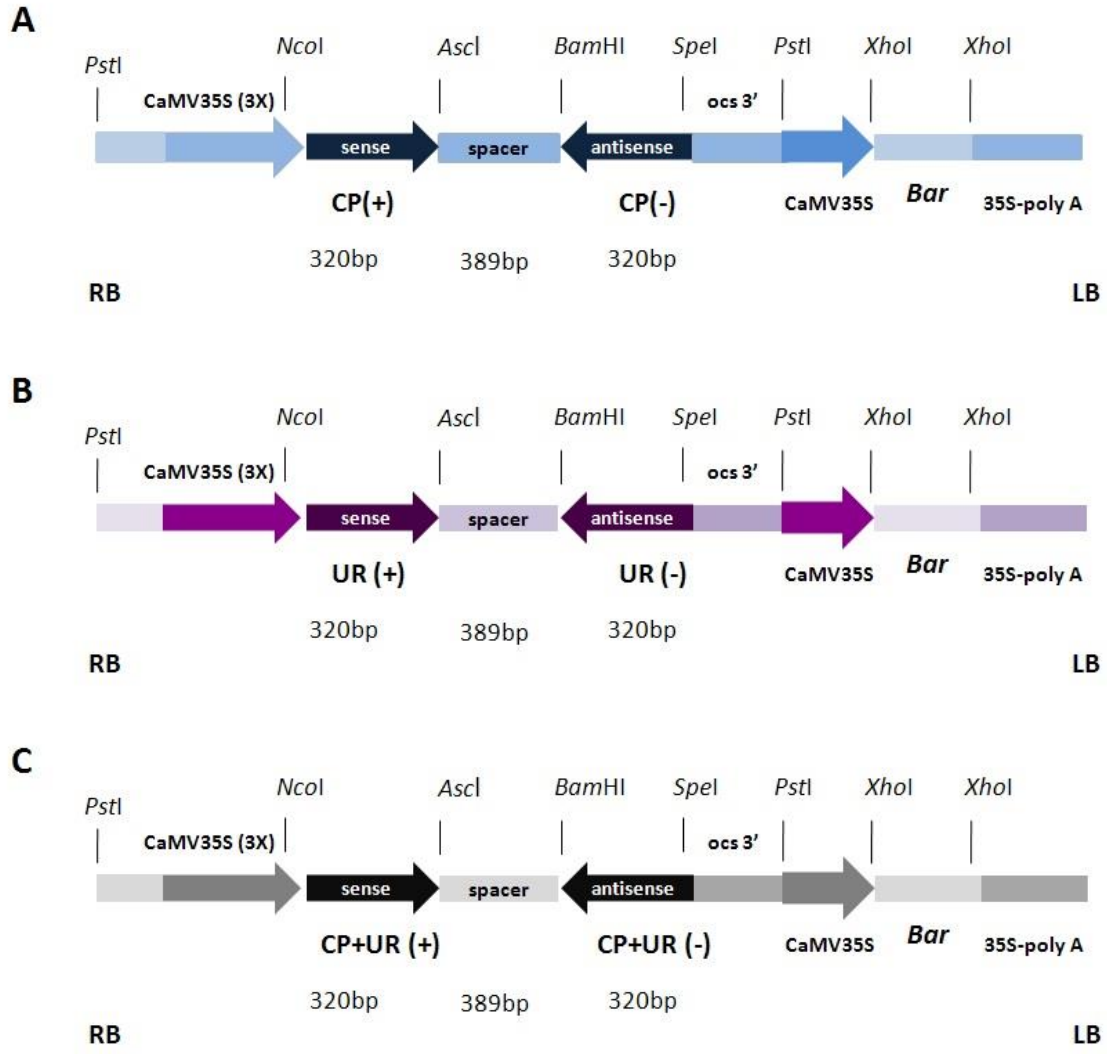

Figure 2.T-DNA fragments map of pCAMBIA3300 carrying PVY inverted repeat. A, coat protein (CP) sequence; B, a segment of coat protein and 3′-untranslated region (CP+UR); C, 3′-untranslated region (UR); RB and LB, respectively, right and left borders of T-DNA; CaMV 35S, cauliflower mosaic virus 35S promoter; OCS 3′, *Octopine synthase*-derived terminator sequence; *Bar*, phosphinothricin resistance gene. *Pst*I, *Nco*I, *Asc*I, *Bam*HI, *Spe*I and *Xho*I are restriction enzymes.

Agroinfiltration and virus inoculation

Potato (*Solanumtuberosum*cv. Marfona) plants were agro-infiltrated with *Agrobacterium tumefacient* AGL1 strain carrying three RNAi constructs (CP, UR and CP+UR) and AGL1 without RNAi vector as control in three replications. Bacteria were grown overnight at 28°C in 50 ml Falcon tubes containing 5 ml of LB medium supplemented with rifampicine (50 mg/l) and kanamycin (50 mg/l). The overnight culture was centrifuged at 4000 rpm for 5 min. The pellets were resuspended in an agroinfiltration buffer containing 10 mM $MgSO_4$ and 10 mM MES (morpholineethanesulfonic acid) (pH 5.5). Plant leaves were injected with the agrobacterial suspension (OD_{600}=0.4) using a needle-free syringe. After agroinfiltration, the plants were grown in 16 h of light at 22°C.

Tobacco plants (*Nicotianatabacum* cv. Samsun) infected with two strains of PVY,

ordinary (PVY^O) and tobacco venial necrosis (PVY^N) were used as PVY infection sources. For inoculums preparation, infected tobacco leaves were rinsed with tap water and weighed. The dry leaves were ground by adding a few drops of inoculation buffer containing 0.1 M sodium/potassium phosphate buffer, pH 7.0, 0.01 M β-mercaptoethanoland carborundum 2% (500 mesh) in a precooled mortar. On obtaining a green paste, more inoculation buffer was added in 2/10 w/v proportion.. About 50 µl of each PVY sap inoculum was rubbed onto the leaves dusted with carborundum (500mesh) 4 days after agroinfiltration. Plants were kept in a greenhouse with 16:8 light cycles at 25°C.

Dot-immunobinding assay (DIBA)

Analysis of viral CP accumulation in plants infected with PVY^O and PVY^N were performed by dot-immunobinding assay. Plant extracts were prepared by homogenizing leaf tissue in phosphate buffered saline(PBS), pH 7.4 (5 ml/g) and centrifuged at 10,000 rpm for 5 min. 10 µl of extracts were gently dropped on polyvinyledenedifluoride (PVDF) membrane (Bio-Rad). The membrane was then air-dried and submerged in skim milk solution (dissolved in PBS 2% w/v) and kept overnight at4°C, followed by washing three times with PBST (PBS+0.1% Tween 20). The membrane was soaked in primary polyclonal antibody (BIOREBA AG, Reinach, Switzerland) specifically diluted to 1:1000 in PBS for 2 h and then washed three times (10 min) in PBST buffer. For PVY detection, the membrane was submerged in enzyme conjugated IgG (W/AP) as a secondary antibody for 2 h and then washed three times (10 min) in PBST buffer. The conjugated alkaline phosphatase activity was visualized directly using the chromogenic substrates BCIP/NBT (bromochloroindolylphosphate + nitrobluetetrazolium) (Roche, Mannheim, Germany).

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

Quantification of PVY in agro-infiltrated and newly emerged leaves was performed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). PVY-specific and alkaline phosphatase conjugated antibodies were purchased from BIOREBA AG, (Reinach, Switzerland). ELISA assays were performed according to manufacturers′ instructions. p-nitrophenylphosphate (pNPP) (BIOREBA AG, Reinach, Switzerland) was used as substrate for immobilized proteins detection in ELISA. Optical density of the reaction products with pNPP as substrate was measured at 405 nm and two measurements were performed for each sample.

Northern blot analysis

Plant total RNA was extracted with TRIZOL (GibcoBRL, Carlsbad, California) from leaves infected 4 and 7 days post-inoculation (dpi). RNA samples (10µg) were electrophoresed on 1.5% agarose gels containing formaldehyde in 1X morphpropanesulphonic acid (MOPS) buffer. Gel redstaining of the agarose gels prior to blotting was done to confirm RNA integrity and loading of similar amounts of RNA. Separated RNAs were transferred to positively charged nylon membrane (Roche, Mannheim, Germany) by capillary blotting and hybridized with PVY PCR-DIG labeled cDNA probe from the PVY-Nib gene (Nuclear inclusion protein) at 52 °C in high SDS hybridization buffer. Posthybridization washes were done twice with 2X saline sodium citrate (SSC) (1X SSC= 0.15Msodium chloride/ 0.015Msodium citrate, pH 7)/ 0.1% SDS and twice with 0.5X SSC/ 0.1% SDS at 50°C. Hybridized probes were visualized using BCIP/NBT substrates (Roche, Mannheim Germany).

Agro-infiltrated potato leaves were analyzed for siRNA accumulation by northern blot hybridization before and after challenge with PVY^N. Total RNAs were isolated (as described above) three days after agroinfiltration and four days after virus inoculation. For siRNA detection, small RNAs were enriched from total RNA by polyethylene glycol (PEG; MW8000) as described by Smith and Eamens (2012) (26). 20 μg of total siRNAs per sample was analyzed by northern blot using a DIG-labeledriboprobeas described by Smith and Eamens (2012) amplified with specific primers for CP+UR construct.

Statistical analysis

The experiments on evaluation of transgenic plants response to different strains of PVY were subjected to analysis of variance (ANOVA) test using a completely randomized design (CRD) with three replications. Means wereseparated by least significant difference (LSD) test at 5% probability level ($P \leq 0.05$). All computationswere performed using SPSS 17.0 statisticalpackage for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Generation of RNAi constructs and transient gene expression

To ensure a broad resistance range against different PVY isolates, initial survey was performed to determine the most conserved regions of the PVY genome. This revealed that the 3′-untranslated region part of the PVY genome is most highly conserved in sequence. Furthermore, three different segments of 320 base fragments were selected from 3′-untranslated region and PVY coat protein. The corresponding cDNA fragments were obtained by RT-PCR from a local PVY strain (PVY isolate ABRII1 Acc. No. EU713856.1). ThecDNA fragment was fused to a 389 bp spacer (linker) in a RNAi destination vector (pGSA1252) under the control of CaMV 35S (3X) promoter. A second copy of cDNA fragment was fused in an inverted orientation to the other side of the spacer fragment. The size of the spacer was chosen on the basis of convenience and stability since shorter spacers have been found to be relatively stable and effective (9). The expression cassette was cloned in the plant transformation vector, pCAMBIA3300 (Fig.2).

Infecting plants with PVY strains and Dot-immunobinding assay

Agro-infiltrated leaves were challenged with PVY strain O and N. The control plants developed systemic symptoms in their upper leaves in a few days, whereas the agro-infiltrated leaves showed no sign of disease development (Fig. 4). At 21 dpi, more than 75% of control plants leaves showed characteristic leaf mottling. The effect was more severe in control plants inoculated with PVY^N than those inoculated with PVY^O.

Plants infiltrated with *Agrobacterium* without RNAi vector displayed disease symptoms in their upper leaves at 7 dpi, whereas all plants that had been agro-infiltrated with the PVYRNAi constructs were free of symptoms or showed symptoms that were delayed three to four weeks compared to the control. Dot blot analysis confirmed the visual observations of PVY CP accumulation in upper leaves at 7 dpi (Fig. 3A). PVY CP was not detectable in plants infiltrated with PVYRNAi constructs (UR, CP, CP+UR) whereas, viral CP was abundant in plants infiltrated with *Agrobacterium* without RNAi vector (Fig. 3A). At 35 dpi,dot blot analysis detected PVY CP in leaves of plants infiltrated withCPconstruct (Fig. 3B).

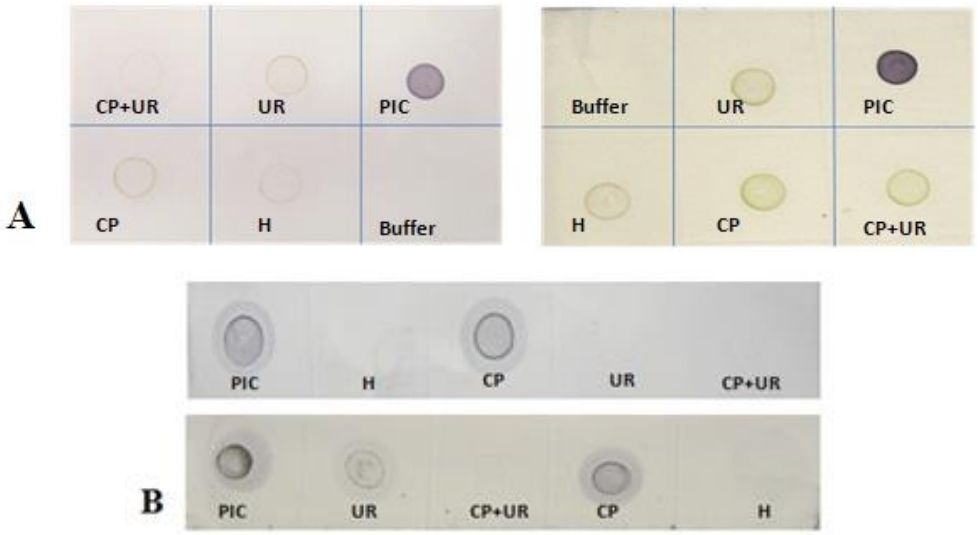

Figure 3. Dot-immunobinding assay for detection of potato virus Y (PVY) in plants infected with PVY^O and PVY^N. A, leaves infected with PVY^O (left) and PVY^N (right) at 7 dpi; B, upper leaves of agro-infiltrated potato plants infected with PVY^O (top) and PVYN (bottom) at 35 dpi; PIC, Potato infected control plant agro-infiltrated with empty vector; H, healthy plants agro-infiltrated with empty vector without virus inoculation; UR and CP, agro-infiltrated leaves with constructs harboring 3′-untranslated region and coat protein regions, respectively.

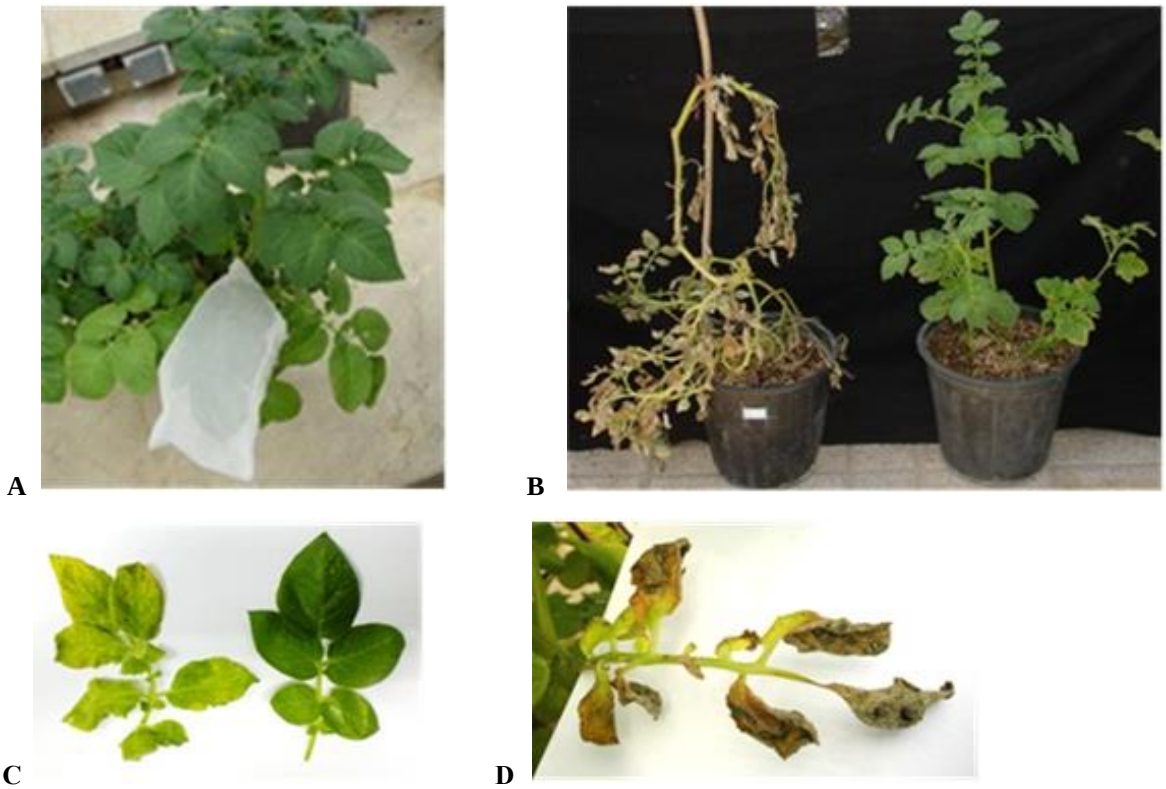

Figure 4. A) RNAi vector agroinfiltration leaves showed no sign of disease development in newly emerged leaves at 30 dpi. B) control plants (left) and CP+UR agro-infiltrated plant (right) inoculated with PVYN. C) control plant leaf showed characteristic leaf mottling (left) and healthy plant leaf emerged from CP agro-infiltrated plant (right) at 7dpi. D) plants agro-infiltrated without RNAi vector displayed disease symptoms in newly emerged leaves at 30 dpi.

Challenge of PVY RNAi constructs with two strains of PVY, O and N, by ELISA test about 21 days post inoculation. CP and UR, potato plants agro-infiltrated with coat protein and 3'-untraslated construct, respectively; PIC, PVY-infected control plant; Ctrl, wild type plant without virus inoculation. Means with similar letters in data table are not significantly different at 5% level of LSD test.

Quantification of PVY infection in virus challenged agro-infiltrated leaves

After inoculation of agro-infiltrated leaves with PVY^N inoculum, the presence of PVY, according to symptoms, was evaluated in samples collected at 7 and 21 dpi, by DAS-ELISA (Table 2). The results showed that only wild type plants were infected with PVY. The highest resistance was obtained from samples infiltrated by CP+UR construct showing high amounts of siRNA, no symptoms and the lowest absorbance in ELISA assay. CP and UR constructs conferred resistance too. Whereas, CP construct showed a little symptom at 7 dpi, newly emerging leaves were free of symptom at 21 dpi (Table 2).

Comparison between the two strains of PVY indicated that PVY^N -infected control plants had higher ELISA value compared to plants infected with PVY^O (Fig. 5). Although, there were no significant differences among RNAi constructs and non-inoculated control plant in response to PVY^O inoculations, there was a significant difference between three RNAi constructs challenged with PVY^N. The lowest ELISA reading was for the CP+UR construct while PVY^N-challenged CP agro-infiltrated leaves showed the highest value.

Table 2. Response of potato leaves agro-infiltrated with different RNAi constructs to PVY^N based on siRNA production, symptoms and DAS-ELISA assays

Constructs	Presence of siRNA	Response to PVY	Mean of ELISA value, inoculated leaves (7dpi)	Mean of ELISA value, upper leaves (21dpi)
CP	Yes	Recovery[a]	0.65	0.31
UR	Yes	Resistance[b]	0.12	0.22
CP+UR	Yes	High resistance[c]	0.09	0.18
PIC	Little	Susceptible[d]	2.02	2.15
Ctrl	NO	No symptom[e]	0.02	0.01

CP and UR, potato plants agro-infiltrated with coat protein and 3'-untranslated region, respectively; PIC, PVY-infected control plant; Ctrl, wild type plant without virus inoculation.
(a): Recovery (plants show a little symptom, but newly emerging leaves were free of symptom at 21 dpi); (b) : Resistance (plants showing no symptom); (c): plants showing no symptoms with the lowest ELISA value; (d): plants showing characteristic leaf mottling; (e): virus non-inoculated control plant.

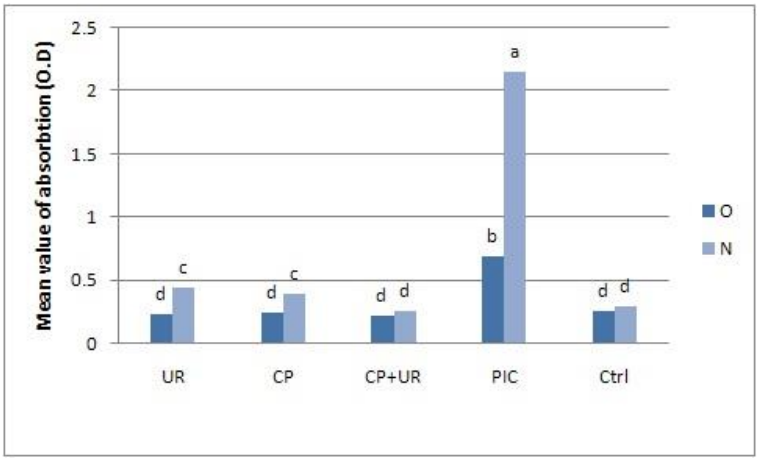

Figure 5.Challenge of PVY RNAi constructs with two strains of PVY, O and N, by ELISA test about 21 days post inoculation. CP and UR, potato plants agro-infiltrated with coat protein and 3'-untraslated construct, respectively; PIC, PVY-infected control plant; Ctrl, wild type plant without virus inoculation. Means with similar letters in data table are not significantly different at 5% level of LSD test.

Detection of viral RNA and expression of siRNA

The presence of PVY RNA was analyzed by northern hybridization in PVY RNA iconstructs agro-infiltrated potato leaves, 7 days post virus inoculation. PVY-Nib segment was detected only in PVY-infected wild type plant as a control (Fig. 6). These results confirmed previous ELISA readings and dot-immunobinding assay data indicating complete resistance in all RNAi constructs at 7 dpi.

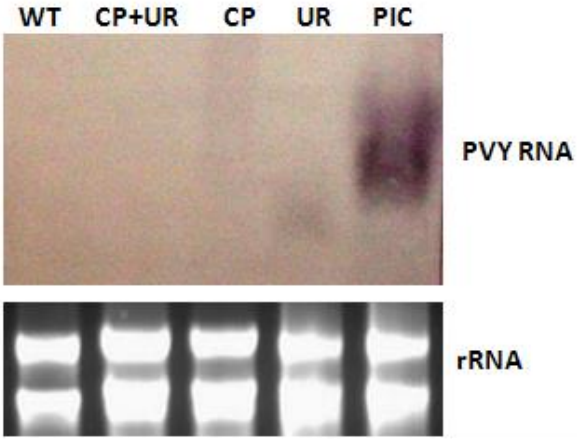

***Figure* 6. Northern hybridization of agro-infiltrated potato leaves for the detection of viral infection 7 days post virus inoculation.**

cDNA from the PVY-Nib gene was used to generate a probe hybridized to RNA from potato plants inoculated with PVY. PIC, PVY-infected wild type plant (control). UR and CP, agro-infiltrated leaves with constructs harboring 3'-untraslated and coat protein regions, respectively; WT: wild type plant without agroinfiltration and virus inoculation. Bottom panel: rRNAs shown as a loading control for the RNA gel blot by staining the gel with Gel Red before transfer.

Dig-labeled riboprobespecific for the PVY CP+UR sequence was used for the three constructs of PVY RNAi. To exclude a potential latent infection with PVY, which might result in virus-derived siRNA that are not of transgene origin, the plants were subjected to DIBA. As expected, no PVY signal was detected in any of the RNAi agro-infiltrated leaves. All the RNAi agro-infiltrated leaves were found to accumulate siRNAs (Fig. 7). After challenge with PVY^N at day 7, the wild type plants and three RNAi constructs agro-infiltrated plants were analyzed for siRNA presence. Northern blot analysis of low molecular weight RNAs showed the accumulation of CP, UR and CP+UR siRNAs before and after virus inoculation. The siRNAs detected mainly belonged to smaller classes of RNAs ideal for RNA silencing (21-23 bp) (7).

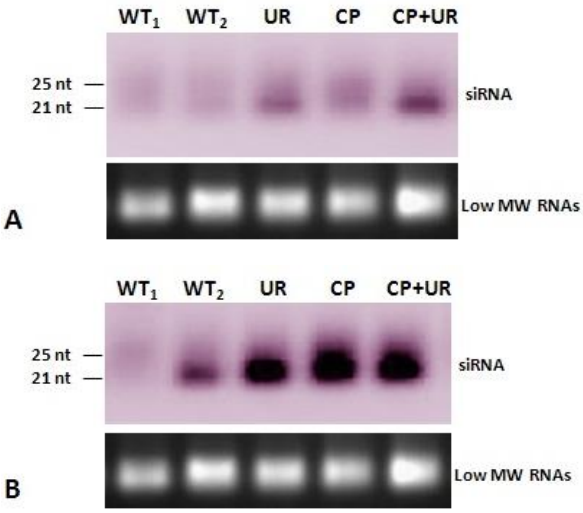

Figure 7. siRNA analysis of transient expression of PVY RNAi constructs.

20 µg of isolated siRNA of leaves was loaded three days after agroinfiltration (A) and four days after virus inoculation (B). WT1: Wild type plant without agro-infiltration. WT2: Wild type plant agro-infiltrated with Agrobacterium without RNAi vector. UR and CP, potato plant agro-infiltrated with constructs harboring 3'-untraslated and coat protein regions, respectively. To ensure equal loading, low molecular weight (MW) RNA of samples was shown by staining the gel with Gel Red before transfer (bottom panel).

Discussion

Post-transcriptional gene silencing has been applied successfully in different plant species to protect them from RNA viruses, and has led to the development of virus resistant crops (10, 12, 15, 18, 19,21, 34,35). The aim of the present work was to engineer strong PVY resistance in potato, an economically

important crop that is severely affected by this virus. To achieve this goal, three constructs containing inverted repeat of different segments of 3′UTR as well as coat protein (CP) region of the virus were produced. Comparison between transient expressions of these constructs indicated that CP+UR was the most efficient RNAi construct to confer PVY resistance in agro-infiltrated plants. Resistance was found to have the form of immunity, since no viral particle could be detected in the upper leaves as shown by DIBA, ELISA assay and northern hybridization. Meanwhile, UR and CP PVY hairpin RNAs generated lower immunity and recovered resistance, respectively. Gaba *et al*. (2010) also reported that transgenic tobacco plants containing 597-nt hairpin RNA construct of the potato virus Y (PVY) replicase sequence were immuned to five potato PVY strains. In contrast, resistance based on transgenic expression of a protein confers relatively broad resistance to related viral strains (Purrahim *et al*., 2005: Niazi *et al*., 2006).

This study has shown that transient expression of an antiviral hairpin RNA by *A. tumefaciens* resulted in its resistance to PVY infection. Several studies have indicated that inverted repeat constructs of transgenes can effectively induce RNA silencing and protect plants against viruses, including mechanically transmitted PVY (3, 27, 28, 31).

Previous researchers have transiently expressed dsRNA of the CP from PVY (31) and AMV, TEV and PMMoV (28). They demonstrated that siRNAs detection derived from the ectopically expressed dsRNA could be used as a prognostic tool to predict virus resistance. Thus, we decided to analyze the expression of new PVY hairpin RNAs from coding (CP) and non-coding region (3′UTR) of PVY genome derived from the transgenes approximately three days after agroinfiltration prior to challenge with PVY^N and 4 dpi.

A unique feature of RNA silencing is the production of siRNAs that act as specific determinants that down regulates gene expression (8). These siRNAs are not only indicative of PTGS but play key roles in PTGS mechanism (33). In this work, all constructs containing inverted repeat of PVY RNA produced significant levels of siRNA before and after inoculation with PVY^N.

PTGS-mediated resistance has potential advantages both in efficacy and biosafety. In most cases, this type of resistance is expressed as immunity. Since little, no protein or RNA is accumulated, essentially no problems related to heterologous encapsidation and recombination between transgene-encoded mRNA and viral RNA would be anticipated. However, as plant viruses have proteins that interfere with PTGS, it is also quite possible that infection with a non-target virus could lead to resistance breakdown, a possibility which requires further investigation.

REFERENCES

1. Baulcombe, D. (2004) RNA silencing in plants. *Nature*., **431**, 356–63.
2. Brodersen, P. and Voinnet, O. (2006) The diversity of RNA silencing pathways in plants. *Trends Genet*., **22**, 268–280.
3. Chen, Y.K., Lohuis, D., Goldbach, R. and Prins, M. (2004) High frequency induction of RNA-mediated resistance against *Cucumber mosaic virus* using inverted repeat constructs. *Mol. Breeding*, **14**, 215-226.
4. Di Nicola-Negri, E., Brunetti, A., Tavazza, M. and Ilardi, V. (2005). Hairpin RNA-mediated silencing of Plum pox virus P1 and HC-Pro genes for efficient and predictable resistance to the virus. *Transgenic Res.*, **14**, 989–94.
5. Fuchs, M. and Gonsalves D. (2007). Safety of virus-resistant transgenic plants two decades after their introduction: lessons from realistic field risk assessment studies. *Annu. Rev. Phytopathol*.,**45**, 173–202.
6. Gaba, V., Rosner, A., Maslenin, L., Leibman, D., Singer, S., Kukurt, E., Shiboleth, Y.M. and Gal-On, A. (2010) Hairpin-based virus resistance depends on the sequence similarity between challenge virus and discrete, highly accumulating siRNA species. *Eur. J. Plant Pathol*.,**128**, 153–164.
7. Hamilton, A., Voinnet, O., Chappell, L. and Baulcombe, D. (2002) Two classes of short interfering RNA in RNA silencing. *EMBO J*., **21**, 4671–9.
8. Hammond, SM., Bernstein, E., Beach, D., Hannon, G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature*, **404**, 293–296.
9. Hirai, S., Kodama, H. (2008) RNAi vectors for manipulation of gene expression in higher plants. *Open Plant Sci. J.*, **2**, 21–30.
10. Kalantidis, K., Psaradakis, S., Tabler, M. and Tsagris, M. (2002) The occurrence of CMV-specific short RNAs in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus. *Mol. Plant Microbe Interact*., **15**, 826–833.
11. Kaniewski, W., Lawson, G., Sammons, B., Haley, L., Hart, J., Delannay, X. and Tumer, N.E. (1990) Field resistance of transgenic Russet Burbank potato to effects of infection by potato virus Xand potato virus Y. *Biotech.*, **8**, 750–754.
12. Krubphachaya, P., Juricek, M. and Kertbundit, S. (2007) Induction of RNA-mediated resistance to papaya ringspot virus type W. *J. Biochem. Mol. Biol*., **40**, 404–411.
13. Lawson, G., Kaniewski, W., Haley, L., Rozman, R., Newell, C., Sanders, P. and Tumer, N.E. (1990) Engineering resistance to mixed virus infection in a commercial potato cultivar: resistanceto potato virus X and potato virus Y in transgenic Russet Burbank. *Biotech*.,**8**, 1277–134.
14. Lindbo, J.A. and Dougherty, W.G. (2005) Plant pathology and RNAi: a brief history. *Annu. Rev. Phytopathol*.,**43**, 191–204.
15. Liu, Y., Schiff, M., Dinesh-Kumar, S.P. (2002) Virus induced gene silencing in tomato. *Plant J*., **31**, 777–786.

16. MacDiarmid, R. (2005) RNA silencing in productive virus infections. *Annu. Rev. Phytopathol*., **43**, 523–44.

17. Maki-Valkama, T., Pehu, T., Santala, A., Valkonen, J.P.T., Koivu, K., Lehto, K. and Pehu, E. (2000) High level of resistance to potato virus Y by expressing P1 sequence in antisense orientation in transgenic potato. *Mol. Breeding*, **6**, 95-104.

18. McCue, K.F., Ponciano, G., Rockhold, D.R., Whitworth, J.L., Gray, S.M., Fofanov, Y. and Belknap, W.R. (2012) Generation of PVY coat protein siRNAs in transgenic potatoes resistant to PVY. *Am. J. Pot. Res*., **89**, 374–383.

19. Missiou, A., Kalantidis, K., Boutla, A., Tzortzakaki, S., Tabler, M. and Tsagris, M. (2004) Generation of transgenic potato plants highly resistant to potato virus Y (PVY) through RNA silencing. *Mol. breeding*, **14**, 185-197.

20. Nelson, R., McCormick, S.M., Delannay, X., Dube, P. and Layton, J. (1988) Virus tolerance, plant growth and field performance of transgenic tomato plants expressing the coat protein from tobacco mosaic virus. *Bio.Technology*, **6**, 403–9.

21. Patil, B.L., Ogwok, E., Wagaba, H., Mohammed, I., Ydav, S.J., Bagewadi, B., Taylor, N.J., Kreuze, J.F., Maruthi, M.N., Alicai, T. and Fauquet, C.M. (2011) RNAi-mediated resistance to diverse isolates belonging to two virus species involved in cassava brown streak disease. *Mol. Plant Pathol*., **12**, 31–41.

22. Powell-Abel, P., Nelson, R.S., De, B., Hoffmann, N. and Rogers, S.G. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science*, **232**, 738–43.

23. Qu, F., Ren, T. and Morris, T.J. (2003) The coat protein of turnip crincle virus suppresses posttranscriptional gene silencing at an early initiation step. *J. Virol*., **77**, 511-522.

24. Sambrook, J. and Russell, D. W. (2001) Molecular cloning. Cold Spring Harbor, New York.

25. Sanford, J.C. and Johnston, S.A. (1985) The concept of parasite-derived resistance deriving resistance genes from the parasite's own genome. *J. Theor. Biol*., **113**, 395–405.

26. Smith, N.A. and Eamens A. (2012) Isolation and detection of small RNAs from plant tissues. In Watson, J.M., Wang, M.B. (eds), Antiviral resistance in plants: methods and protocols. *Methods Mol. Biol*., **894**, 155–172.

27. Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P., Green, A. and Waterhouse, P.M. (2000) Total silencing by intron-spliced hairpin RNA. *Nature*, **407**, 319-320.

28. Tenllado, F. and Díaz-Ruíz, J.R. (2001) Double-Stranded RNA-Mediated Interference with Plant Virus Infection. Journal of virology,75.

29. Tepfer, M. (2002) Risk assessment of virus-resistant transgenic plants. *Annu. Rev. Phytopathol*.,**40**, 467–91.

30. Thomas, C.L., Leh, V., Lederer, C. and Maule, A.J. (2003) Turnip crincle virus coat protein mediates suppression of RNA silencing in *Nicotiana benthamiana*. *Virology*, **306**, 33-41.

31. Vargas, M., Martínez-García, B., Díaz-Ruíz, J.R. and Tenllado, F. (2008) Transient expression of homologous hairpin RNA interferes with PVY transmission by aphids. *Virology J.*, **5**, 42.

32. Voinnet, O. (2001). RNA silencing as a plant immune system against viruses. *Trends Genet.*,**17**, 449–59.

33. Waterhouse, P.M., Wang, M.B. and Lough, T. (2001) Gene silencing as an adaptive defence against viruses. *Nature*, **411**, 834–842.

34. Yadav, S.J., Ogwok, E., Wagaba, H., Patil, B.L., Bagewadi, B., Alicia, T., Gaitan-Solis, E., Taylor, N.J. and Fauquet, C.M. (2011) RNAi mediated resistance to cassava brown streak Uganda virus in transgenic cassava. *Mol. Plant Pathol*.,**12**, 677–687.

35. Zhang, P., Vanderschuren, H., Futterer, J. and Gruissem, W. (2005) Resistance to cassava mosaic disease in transgenic cassava expressing antisense RNAs targeting virus replication genes. *Plant Biotechnol. J.*, **3**, 385–397.