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# Characteristics of Resistance to Potato Virus Y in Transgenic Tobacco Plants Mediated with Complimentary DNA (cDNA) of PVY Replicase Mutant Genes

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**ABSTRACT : This study was conducted to develop a resistant tobacco against potato virus Y (PVY) by transformation of the plants with genetically engineered viral genes. The complimentary DNAs (cDNAs) of potato virus Y-necrosis strain (PVY-VN) replicase mutant genes (3'-deleted, 5'-deleted and ADD-mutant NIbs) were synthesized through RT-PCR by using purified PVY-VN RNA and synthesized primers, and cloned in the sense orientation into a plant expression vector (pMBP1). The cDNAs of the genes were transferred into *Agrobacterium tumefaciens* LBA 4404, and then transformed into tobacco (*Nicotiana tabacum* cv. Burley 21) plants. Regenerated plants were tested for PVY resistance by inoculation test; 13 transgenic plants including 7 for 3'-deleted NIb, 3 for 5'-deleted NIb, and 3 for ADD-mutant NIb appeared to be resistant at 4 weeks after inoculation with PVY-VN. Among the 13 transgenic tobacco plants, 8 plants had no symptom up to 14 weeks after inoculation. The progenies ($T_1$) from self-fertilization of the transgenic lines varied 0.0% to 81.2% in their resistance (% of resistant plants). The analysis of NIb-3'deleted, -5'deleted and -ADD mutant in the $T_1$ plants by polymerase chain reaction (PCR) showed that NIb-3'deleted, -5'deleted and -ADD mutants were detected in all of the resistant plants. These results suggest that the PVY resistance was inherited in the $T_1$ generation.**

**Key words : PVY, replicase mutant genes, transgenic tobacco plants, PCR**

Potato virus Y (PVY) distributes worldwide and causes severe damages in a wide range of crops (Agrios, 1988). Tobacco is an important host of PVY, and the virus occurs frequently where tobacco plants are growing (Lucas, 1975). In Korea PVY occurs frequently both in burley tobacco and flue-cured tobacco fields, and in some areas where potato is collectively growing, tobacco plantations are devastated by PVY infection. For example, in a central region of the country in which potato had been rarely planted previously, PVY occurrence increased drastically after a new potato cultivar was introduced and largely grown, and in the second year more than 90 % of the tobacco plants in that area were infected with PVY around the harvest time.

PVY is mostly transmitted into tobacco plants by green peach aphids under natural conditions. Control practices of infection or spread of a virus disease in a crop usually rely on the elimination of

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the aphids such as insecticide spray and prevention of their movement from field to field (Lucas, 1975). However, these practices do not give sufficient control efficacies for PVY infection. Use of tobacco cultivars resistant to PVY may be one of the most reliable control methods against PVY.

In recent years, recombinant DNA techniques have been applied to develop resistant transgenic plants mainly transformed with viral genes such as coat protein genes, replicase genes, and satellite RNA (Baulcombe, 1996; Lindbo and Dougherty, 1992; Macfarlane and Davies, 1992). So far much attention has been made on coat protein (CP)-mediated resistance, while relatively little study has been conducted on viral genes encoding replicases, deleted- and mutant-replicase genes. This experiment was focused on the transformation of tobacco plants with viral deleted- and mutant-replicase genes to examine characteristics of the resistance, which in turn may lead additional strategies for developing disease resistant plants.

## MATERIALS AND METHODS

**Bacterial strain and plasmid.** The bacterial strain used in these experiments is *E. coli* HB101 [*supE44 hsdS20(rB⁻mB⁻) recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl5 mtl1*] for transformation and plasmid conservation. Plasmid pUC19 was used for cloning and sequence analysis.

**Enzymes and chemicals.** All the materials required for synthesis of cDNA library, [α-$^{32}$P] dCTP and [α-$^{35}$S]dATP were purchased from Amersham International plc, UK. Restriction enzymes *Eco*RI, *Hin*dIII and *Sma*I were purchased from KOSCO, Korea. DNA sequencing kit with sequenase version 2.0 T7 DNA polymerase was purchased from United States Biochemical (USB). Synthetic oligomers were from Korea Biotech. Other chemicals were from Sigma Chemical Co., USA.

**Transformation of tobacco plants with viral genes and plant regeneration.** The procedures for the transformation with PVY-VN NIb-3′ deleted, -5′deleted and -ADD mutant cDNAs and regeneration of tobacco plants (*Nicotiana tabacum* cv. Burley21) are shown in Figure 1.

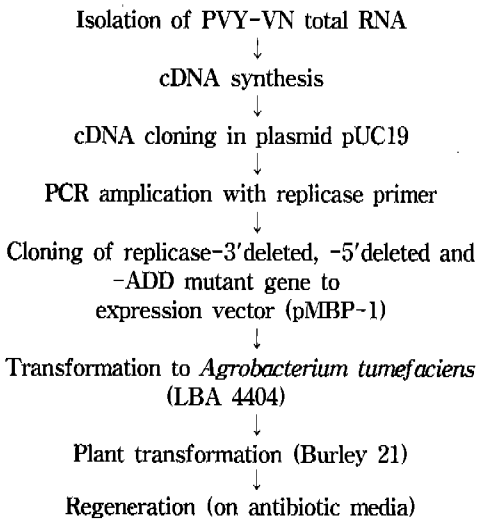


Fig. 1. Procedure for genetic engineering of tobacco plants.

**RNA isolation from PVY-VN particles.** PVY particles were isolated from inoculated leaves of *Nicotiana tabacum* cv. Burley 21. After 2 weeks of mechanical inoculation with PVY-VN, leaves were homogenized, the virus was precipitated by carrying out two times of low speed centrifugation, and the precipitate was purified by cesium chloride (38%) density gradient centrifugation. The viral RNA used in this study was isolated from the purified virus suspended in TNE buffer (10mM Tris-HCl, pH 7.5, 100mM NaCl, 10mM $Na_2EDTA$) added SDS to 4%(W/V) and equal volume of TNE-saturated phenol and then the mixture was shaken intermittently on ice for 10 min, centrifuged, recovered aqueous phase and re-extracted with the TNE-saturated phenol. The RNA was concentrated by ethanol precipitation with 1/10 volume of 3M sodium acetate (pH 5.5).

**cDNA synthesis and cloning.** Reaction conditions for cDNA synthesis were those from the Amersham cDNA synthesis system. Viral RNA (4 μg) isolated as above was reverse transcribed into cDNA primed with oligo(dT) during first strand

synthesis, and RNase-H-digested template was the primer for second strand synthesis. Reactions were quantified by the incorporation of [α-$^{32}$P]dCTP. The product was treated with $T_4$ DNA polymerase and the blunt-ended size distribution was analysed on alkaline agarose gels. The double-stranded cDNA products were then ligated into *Sma*I site in pUC19.

**Subcloning the PVY-VN NIb cDNA clone into plant expression vector.** By using the primer set (5′-primer; GCAGGATCCGACA ATG GGC TAA GCA TTC TGC ATG GAT G/ 3′-primer; GCAGGATCC TCA TTG ATG GTG CAC TTC ATA AGA) polymerase chain reaction (PCR) (at 94℃ for 30 sec, at 60℃ for 5 min, at 72℃ for 1 min, 30 cycle) were carried out to clone NIb-3′deleted, -5′deleted and -ADD mutant cDNA fragment. Sequencing was carried out to exclude any artefacts from PCR analysis. For plant transformation, verified cDNA clone of PVY-VN NIb was introduced into plant expression vector, pMBP1 as *Bam*HI fragment. The orientation of the inserts, antisense or sense, was confirmed by *Sma*I and *Bgl*II restriction enzyme analysis.

**Plant transformation and regeneration.** The plasmids (pMBP-1) containing the PVY-VN NIb-3′deleted, -5′deleted and -ADD mutant cDNA clone were transferred from *E. coli* HB101 into *Agrobacterium tumefaciens* strain LBA4404 by direct DNA uptake method (An, 1987). The structure of the vector mobilized into *Agrobacterium* was verified by restriction digestion of purified *Agrobacterium* plasmid DNA. Tobacco (Burley 21) transformation was done essentially according to Horsch *et al.* (1985). Induction of callus and shoot formation were done on solid MS medium (Murashige and Skoog, 1962) supplemented with 2.0 mg/l benzylamino purine and 200 mg/l kanamycin sulfate as a selection agent. For root induction kanamycin-resistant regenerated shoots were transferred to hormone-free MS medium containing 100㎎/l kanamycin. After root development (2 to 3 weeks), plantlets were transferred to potting soil and developed into the whole plant in a greenhouse. Transgenic plants were fully grown in a greenhouse and self-fertilized to produce seeds. The first (T1) progenies were grown and tested for resistance to PVY and viral infection in the greenhouse.

**Detection of genes introduced in transgenic tobacco plants**

**Polymerase chain reaction analysis.** Two specific oligonucleotides derived from *npt*II gene were used as primers for PCR. One (5′-GAGGC TATTCGGCTATGACTG-3′) is located in the position from 201 to 221 of the *npt*II gene, and the other (5′-ATCGGGAGCGGCGA TACCG TA-3′) in the position from 900 to 880 (Beck *et al.*, 1982). Otherwise the same primer set for NIb-3′deleted, -5′deleted and -ADD mutant cDNA cloning was used for verification of transgenic tobacco plants. Genomic DNA was amplified by the modified procedure of Edwards *et al.* (1991). PCR reaction was carried out in 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 50 μg/ml BSA, 0.001% gelatin, 200 μM each of dATP, dCTP, dTTP, dGTP, 2 units of *Taq* DNA polymerase (Korea Biotech.) and 50 pM of each oligonucleotide primer. PCR was performed in a thermal cycler (Perkin Elmer Cetus) for 35 cycles, with each cycle consisting of 94℃ for 1 min to denature the template, 55℃ for 1 min for primer annealing and 72℃ for 2 min for polymerization. At the end of 35 cycles, samples were incubated for 7 min at 72℃ and kept at 4℃ prior to gel analysis.

**Assays of viral infection and resistance of transgenic plants to PVY-VN**

**Viral symptom development assays.** Tobacco plants about 3 weeks after transfer to potting soil (at the stage of 5 or 6 true leaves) were used for assays of virus infection. PVY-VN was used to inoculate the transgenic plants and non-transformed plants. Two or three leaves from the non-transformed and transgenic plant lines were dusted with carborundum (500 mesh) and mechanically inoculated with PVY-VN at an approximate concentration of 2 μg/ml in 10 mM phosphate buffer, pH 7.2. After the inoculated leaf surface were rinsed with tap water, and the plants

were grown in the greenhouse at 22~30℃. Symptom development was monitored on a daily basis after inoculation. *Nicotiana tabacum* cv. Xanthi-nc was used for reinoculation experiment with sap prepared from the inoculated and upper leaves of the inoculated ones in the transgenic plants.

ELISA and Serological test. Detection of viral infection was also assayed serologically by using direct double sandwich ELISA method described by Clack and Adams(1977) and capillary tube test with reaction of plant sap to PVY-VN antiserum. The optimum dilutions of antiserum and plant sap were always checked before testing serological reactions.

## RESULTS AND DISCUSSION

The main goal of this study was to analyze and introduce PVY-VN NIb-3'deleted, -5'de- leted and -ADD mutant cDNA clone into the cultivating tobacco plant (Burley 21) and develop virus-resistant transgenic tobacco plants. Intensive bioassay and segregation analyses up to $T_1$ generation of some transgenic lines were carried out and confirmed stable inheritance of introduced genetic clone in the transgenic plants.

**Analysis of PVY-VN NIb-3'deleted, -5'de-leted and -ADD mutant cDNA cloned and recombination in plant expression vector.**

Nucleotide sequencing of PVY-VN NIb-3'deleted, -5'deleted and -ADD mutant cDNA was carried out on both orientations with sequenase using universal primers and synthetic oligomers, according to the protocol suggested by the supplier. The sequence of nucleotides coding for the entire NIb was determined with the predicted amino acid sequences(Cho *et al*, 1994). The PVY-VN NIb is 1,557 nucleotides long expressing 57 kDa protein. Overall comparison of NIb as sequences of PVY-VN with those of other PVY strains shows 92 to 93% identity. The NIb/CP polyprotein cleavage site of PVY-VN was found to be well conserved as those in other PVY strains (Robaglia *et al.*, 1989). The conserved amino acid sequences surrounding cleavage site of PVY are SYEVHHQ /A(or G)NDTID. The GDD motif of NIb known to be a viral polymerase domain is also conserved in PVY-VN sequence.

Comparison of the nucleotide sequences with other PVY cDNA clones listed in the GenBank data base showed that the cDNAs have not been previously reported. In this experiment, the cloned NIb-3'deleted, -5'deleted and -ADD mutant cDNA was recombined with the direction of sense to plant expression vector pBMP-1, using 35S promotor doubly to enhance the expression of the inserted gene in the plant system (Fig. 2).

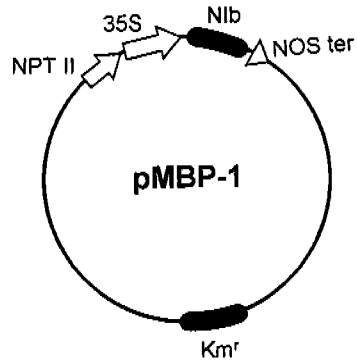


Fig. 2. Plant expression vector (pMBP-1) inserted with PVY-VN NIb-3'deleted, -5'deleted and -ADD mutant cDNA (ca. 1.5 kb). Plasmid size: 15.5 kb.

**Resistance and gene introduction in regenerated transgenic plants.**

The putative transgenic plants, which were selected on kanamycin media (kanamycin-resistant, $Km^R$), were tested for their resistance to PVY-VN. Out of regenerated plants with sense-oriented gene insertion, 13 transgenic plants including 7 for 3'-deleted NIb, 3 for 5'-deleted NIb, and 3 for ADD-mutant NIb had no symptom resistance at 4 weeks after PVY-VN inoculation while normal Burley 21 plant showed susceptible vein necrosis symptoms (Table 1). Out of the 13 resistant plants, five plants mild vein necrosis PVY symptoms appeared 14 weeks after PVY-VN inoculation. This result indicates that symptom development was delayed in the some transgenic plants. But the eight plants with no symptom were

reinoculated with PVY-VN, and symptom development was examined up to 14 weeks after inoculation. These plants were self-fertilized, and the resistance of the next generations was further characterized.

Virus was detected by both serology and inoculation test in all the inoculated leaves 2~3 weeks after PVY-VN inoculation. And the selected 8 transgenic plants with no PVY symptom were tested for the viral presence in the upper 5 leaves of the inoculated ones by serology and bioassay. But virus was not detected by both serology and inoculation test in all the upper leaves 8 and 10 weeks after PVY-VN inoculation. However the other 5 plants showed mild vein necrosis PVY symptoms at 14 weeks after PVY-VN inoculation. This indicates that the resistant transgenic plants may be infected with the virus on the inoculated leaves; however, they may resist the systemic viral infection (Table 1).

**Evaluation of the $T_1$ generation segregation pattern in a transgenic plant line (3′deleted-, 5′deleted- and ADD mutant-NIbs).**

Tobacco plants of the $T_1$ generation derived from 13 lines selected in the regenerated plants ($T_0$ generation) were inoculated with PVY-VN and symptom development of PVY was observed(Table 2). After 2 weeks, all of the untransformed Burley 21 tobacco plants were severely infected, showing susceptible PVY-VN symptoms (vein necrosis), while the transgenic lines varied in the degree and percentage of symptom development; i.e., no symptom, mild (delayed) symptoms (appearing on small portions of leaves) and susceptible (severe) symptoms. Out of 7 lines tested the 3′ deleted NIb transgenic line, one line (N3′(S)-17) appeared to be susceptible to PVY-VN as untransformed tobacco

Table 1. Resistance of regenerated transgenic plants to PVY infection

| Gene | Plant line | *Symptom of plants | | | |
|---|---|---|---|---|---|
| | | Weeks after PVY-VN inoculation | | | |
| | | 4 | 8 | 10 | 14 |
| NIb-3′deleted | N3′(S)-1 | NS | NS (−) | NS (−) | NS |
| | N3′(S)-7 | NS | NS (−) | NS (−) | NS |
| | N3′(S)-9 | NS | NS (−) | NS (−) | mVN |
| | N3′(S)-10 | NS | NS (−) | NS (−) | NS |
| | N3′(S)-14 | NS | NS (−) | NS (−) | NS |
| | N3′(S)-17 | NS | NS (−) | NS (−) | NS |
| | N3′(S)-18 | NS | NS (−) | NS (−) | mVN |
| NIb-5′deleted | N5′(S)-9 | NS | NS (−) | NS (−) | NS |
| | N5′(S)-12 | NS | NS (−) | NS (−) | NS |
| | N5′(S)-22 | NS | NS (−) | NS (−) | mVN |
| NIb-ADD mutant | N ADD(S)-1 | NS | NS (−) | NS (−) | mVN |
| | N ADD(S)-6 | NS | NS (−) | NS (−) | NS |
| | N ADD(S)-14 | NS | NS (−) | NS (−) | mVN |
| Control | Burley 21 (PVY-VN inoculated) | VN | VN | VN | VN |
| | Burley 21 (Healthy normal) | NS | NS | NS | NS |

* NS: No symptom on main and sucker leaves, mVN: mild vein necrosis, VN: vein necrosis
− in the pararenthes indicates the negative reaction of plant sap to PVY-VN antiserum by serology test.

plants, another four lines (N3′(S)-1, -7, -9 and -18) had less than 42 % resistant plants, but in the other two lines (N3′(S)-10 and -14) had the resistant plants over 60 % (Table 2). Out of 3 lines tested the 5′ deleted NIb transgenic line, one line (N5′(S)-22) had less than 23 % resistant plants, but the other two lines had the resistant plants over 70 %. And Out of 3 lines tested the ADD mutant NIb transgenic line, two lines (ADD(S)-1, -14) had less than 26 % resistant plants, but in the other one line (ADD(S)-6) had the resistant plants over 58 % after 2 weeks of inoculation (Table 2). Three progenies were chosen among the 13 virus resistant plant lines for further analysis of resistant characteristics for the next generation.

PCR was used to demonstrate whether T-DNA was present in the putative $Km^R$ transgenic tobacco plants. Two specific primers derived from *npt*II gene sequences were used to detect a 0.7 kb fragment. And other two specific primers used for amplification of 1.5 kb replicase deleted and mutant genes were also used to amplify the DNA by PCR for detection of the presence of the mutant genes in the $T_1$ transgenic plants. The amplified DNA samples were then fractionated by electrophoresis on a 0.8 % agarose gel. Amplified product could be detected from transformed tobacco plants, but not from non-transformed plants (data not shown). This result was obtained reproducibly, sugesting that an *npt*II gene and PVY-VN replicase deleted and mutant genes were inherited in the $T_1$ generation of the transgenic plants.

The selected plants which showed no symptom 2~3 weeks after inoculation were also examined for the T-DNA insertion, and the amplified product by PCR was also detected in all of the plants. PCR analysis of all the 10 resistant progeny of NIb-3′deleted (N3′-10-1~10), 18 resistant progeny of NIb-5′ deleted (N5′-9-1~18) and 10 resistant progeny of NIb-ADD mutant (ADD(S) 6-1~10) showed positive and these confirm the gene transmission to the next generation(Fig. 3, 4 and 5).

Fifteen to 50 plants with no symptom at 2

Table 2. PVY resistance of the 1st progenies ($T_1$) of the transgenic plant 2 weeks after PVY-VN inoculation

| Plant line | No. plants tested | No. plants against PVY | | |
|---|---|---|---|---|
| | | Highly resistant[1] | Moderately resistant[2] | Susceptible |
| N3′(S)-1 | 142 | 21 (14.7) | 38 (26.8) | 83 (58.5) |
| N3′(S)-7 | 108 | 15 (13.9) | 6 (5.5) | 87 (80.6) |
| N3′(S)-9 | 108 | 22 (20.4) | 11 (10.2) | 75 (69.4) |
| N3′(S)-10 | 140 | 54 (38.6) | 38 (27.1) | 48 (34.3) |
| N3′(S)-14 | 142 | 39 (27.5) | 47 (33.1) | 56 (39.4) |
| N3′(S)-17 | 144 | ( 0 ) | ( 0 ) | 144 (100) |
| N3′(S)-18 | 144 | 14 (9.7) | 9 (6.3) | 121 (84.0) |
| N5′(S)-9 | 144 | 116 (80.5) | 1 (0.7) | 27 (18.8) |
| N5′(S)-12 | 72 | 27 (37.5) | 25 (34.7) | 20 (27.8) |
| N5′(S)-22 | 108 | 21 (19.4) | 3 (2.8) | 84 (77.8) |
| ADD(S)-1 | 143 | 30 (21.0) | 7 (4.9) | 106 (74.1) |
| ADD(S)-6 | 179 | 82 (45.8) | 22 (12.3) | 75 (41.9) |
| ADD(S)-14 | 108 | 18 (16.6) | 2 (1.9) | 88 (81.5) |
| Burley 21 | 144 | ( 0 ) | ( 0 ) | 144 (100) |

1. No symptom was developed on the resistant plants.
2. Symptom was observed mild vein necrosis.
Number in the parenthesis is the percentage of the plants against the PVY.

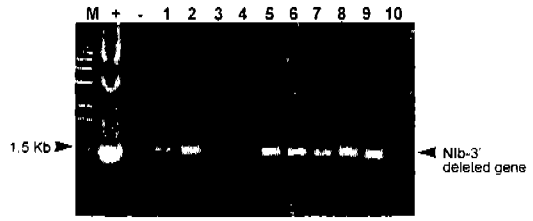


Fig. 3. Detection of PVY-VN NIb-3'deleted gene (1.5 kb) in the $T_1$ transgenic plants by PCR analysis. M: 1 kb ladder, +: positive control, -: negative control (a healthy untransformed plant), 1-10: resistant transgenic plants.

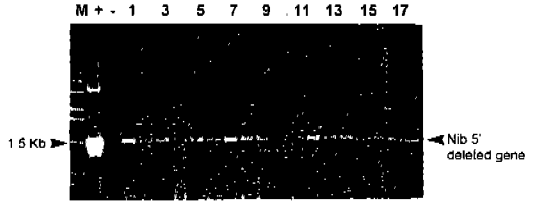


Fig. 4. Detection of PVY-VN NIb-5'deleted gene (1.5 kb) in the $T_1$ transgenic plants by PCR analysis. M: 1 kb ladder, +: positive control, -: negative control (a healthy untransformed plant), 1-18: resistant transgenic plants.

weeks after inoculation were selected from 5 lines that had a good resistance, and symptom development was examined up to the seedharvesting time. In all the 4 lines except N3'(S)-1 plant line, no

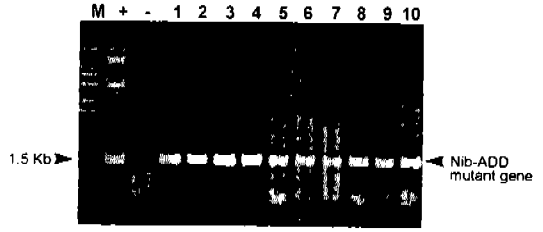


Fig. 5. Detection of PVY-VN NIb-ADD mutant gene in the $T_1$ transgenic plants by PCR analysis. M: 1 kb ladder, +: positive control, -: negative control (a healthy untransformed plant), 1-10: resistant transgenic plants.

plant showed susceptible symptoms, and most of the plants remained symptomless (Table 3). These suggest that the transgenic plants mediated with PVY-VN NIb- 5'deleted and ADD-mutant genes may express durable resistance to PVY-VN. In ELISA analysis, these plants did not showed the virus infection in all leaves 10 weeks after PVY-VN inoculation and reinoculation of the sap prepared from these plants on Xanthi-nc tobacco also failed to induce any PVY symptom at all (Table 4). Thus, these $T_1$ plant lines showed almost a perfect resistance against PVY-VN infection.

## CONCLUSION

Regenerated tobacco plants (*Nicotiana tabacum*

Table 3. PVY resistance of the 1st progenies ($T_1$) of the transgenic plant 4 weeks after PVY-VN inoculation,

| Plant line | No. plants tested | Plants against PVY(%) | | |
|---|---|---|---|---|
| | | Highly resistant[1] | Moderately resistant[2] | Susceptible |
| N3'(S)-1 | 15 | 20 | 80 | 0 |
| N3'(S)-10 | 30 | 100 | 0 | 0 |
| N5'(S)-9 | 50 | 100 | 0 | 0 |
| N5'(S)-12 | 20 | 100 | 0 | 0 |
| ADD(S)-6 | 30 | 100 | 0 | o |

1. No symptom was developed on the resistant plants.
2. Symptom was observed mild vein necrosis.

Table 4. PVY infection of the 1st progenies ($T_1$) of the resistant transgenic plants 10 weeks after PVY-VN inoculation

| Plant line | Plant Symptom | ELISA | Bioassay |
|---|---|---|---|
| N3′(S)-1-1 | mild vein necrosis | + | + |
| N3′(S)-1-6 | Healthy | − | − |
| N3′(S)-1-7 | mild vein necrosis | + | + |
| N3′(S)-1-8 | mild vein necrosis | + | + |
| N3′(S)-1-9 | Healthy | − | − |
| N3′(S)-1-10 | Healthy | − | − |
| N5′(S)-9-2 | Healthy | − | − |
| N5′(S)-9-4 | 〃 | − | − |
| N5′(S)-9-6 | 〃 | − | − |
| N5′(S)-9-7 | 〃 | − | − |
| N5′(S)-9-11 | 〃 | − | − |
| ADD(S) 6-1 | Healthy | − | − |
| ADD(S) 6-2 | 〃 | − | − |
| ADD(S) 6-3 | 〃 | − | − |
| ADD(S) 6-4 | 〃 | − | − |
| ADD(S) 6-6 | 〃 | − | − |
| ADD(S) 6-8 | 〃 | − | − |
| Burley 21 (healthy normal) | Healthy | − | − |
| Burley 21 (PVY-VN inoculated) | Vein Necrosis | + | + |

cv. Burley 21) were tested for PVY resistance by inoculation test; 13 transgenic plants including 7 for 3′-deleted NIb, 3 for 5′-deleted NIb, and 3 for ADD-mutant NIb appeared to be resistant at 4 weeks after inoculation with PVY-VN. Among the 13 transgenic tobacco plants, 8 plants had no symptom up to 14 weeks after inoculation. The progenies ($T_1$) from self-fertilization of the transgenic lines varied 0.0% to 81.2% in their resistance (% of resistant plants). The analysis of NIb-3′deleted, -5′deleted and -ADD mutant in the $T_1$ plants by polymerase chain reaction showed that NIb-3′deleted, -5′deleted and -ADD mutant were detected in all of the resistant plants.

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