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Resistance to Potato Virus Y Conferred by PVY Replicase Gene Sequence in Transgenic Burley Tobacco

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ABSTRACT : The complementary DNA (cDNA) of potato virus Y- vein necrosis strain (PVY-VN) replicase gene (NIb) was transformed into tobacco (*Nicotiana tabacum* cv. Burley 21) plants. Out of 25 putative transformants regenerated, 3 were resistant to PVY-VN, one highly resistant plant with no symptom until seed harvest time and the other two with mild chlorotic spot symptoms at late stages after infection. No symptom was observed in the highly resistant plant, while mild vein necrotic symptoms were developed on suckers of the moderately resistant plants after seed harvest time. In the first generation (T_1) via self fertilization, resistance to susceptibility frequency in transgenic plants from the highly resistant transformant was about 3 : 1, while it was lowered much (about 1:2 and 1:19) in T_1 of the moderately resistant transformants. In the second generation (T_2) of the highly resistant plant, resistance frequencies were similar to T_1, but resistance levels varied greatly and appeared to be decreased.

Key words **: potato virus Y, viral replicase gene, transgenic tobacco plants, resistance.**

There are a variety of ways to control plant viruses, among which use of resistant plants is regarded as one of the most efficient and reliable control methods. In the breeding of resistant crops, however, resistant gene sources have not been readily available for the control of many viruses. Also very few resistant cultivars bred have been extensively cultivated for the control of virus diseases because of inferior genes linked to target genes, rendering other agronomic traits such as qualitative and quantitative yield worse.

Potato virus Y (PVY) is distributed worldwide and causes severe economic losses in leaf tobacco production. PVY is transmitted by aphids in nature, and control methods other than resistance are related to elimination and reduction of the insect vectors. In Korea, PVY occurs in wide areas of tobacco plantations, especially in burley tobacco fields. Various cultural and chemical methods to control the insect vectors have been applied to be unsuccessful in fully controlling PVY infection, and thus resistance is the most practical way to control the virus (Burk and Chaplin, 1980; Chae et al., 1994).

Several tobacco cultivars or lines resistant to PVY have been developed by breeding, which have either inferior leaf tobacco quality or susceptibility to other diseases (Gupton, 1980; Miller, 1987). Recently new burley tobacco cultivars were developed in Korea, which had PVY resistance and

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equal leaf tobacco production and quality to Burley 21 (Jung *et al.*, 1994; 1997).

Development in recombinant DNA techniques in recent years has made it possible to incorporate only target genes into plants for expressing new single traits including disease resistance. However, no structural gene responsible for virus resistance has been fully characterized and identified, so that the techniques have been limited in the practical application for breeding resistant plants at present. Alternatively, virus resistance has been obtained by expressing sequences derived from viral genomes in transgenic plants. Viral genes that have been used in plant transformation for virus resistance were satellite RNAs, antisense RNAs, the whole or portion of viral replicase, and viral coat protein (CP) genes (Beachy, 1993).

Compared to CP-mediated virus resistance, few examples of replicase-mediated resistance have been reported, and thus relatively little is known about the resistance characteristics of the latter. Thus, this experiment was focused on the transformation of tobacco plants with viral replicase gene to examine characteristics of the resistance, which may in turn lead to the additional strategies for developing PVY resistant plants.

MATERIALS AND METHODS

Plant transformation and regeneration. Bacterial strains and plasmid, enzymes and chemicals, and all procedures for viral RNA isolation, and cDNA synthesis and cloning used in plant transformation were same as those of the previous experiment using a flue-cured tobacco cultivar (Park et al., 1997). For gene construction, the cloned NIb cDNA was recombined with the direction of sense to plant expression vector pMBP-2 with 35S double promoter to enhance the expression of the inserted gene in the plant system (Fig. 1).

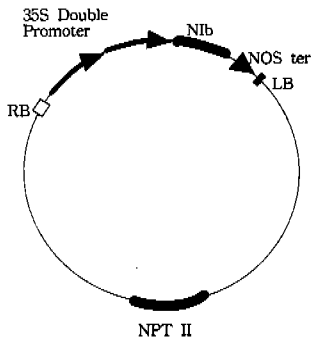

Fig. 1. A schematic diagram of the plant expression vector, pMBP-2, inserted with PVY-VN replicase cDNA.

The constructed gene was transferred into *Agrobacterium tumefaciens* strain LBA 4404 by direct DNA uptake. The structure of the vector mobilized into *Agrobacterium* was verified by restriction enzyme analysis of purified *Agrobacterium* plasmid DNA. Tobacco (cv. Burley 21) transformation was performed by inoculating the transformed *Agrobacterium* strain (Horsch *et al.*, 1985). Calli and shoots were formed 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 supplemented with 100 mg/l kanamycin. After root development (2 to 3 weeks later), plantlets were transferred to potting soil and developed into the whole plants in a greenhouse. Transgenic plants were fully grown in the greenhouse and self-fertilized to produce seeds.

Assays of viral infection and resistance of transgenic plants to PVY-VN. Tobacco plants about 3 weeks after transfer to potting soil (at the stage of 4 true leaves) was used for assays of virus infection. PVY-VN was used to inoculate the transgenic plants and non-transformed Burley 21 tobacco plants. The non-transformed and transgenic plants were dusted with carborundum (500 mesh) on 2 leaves and mechanically inoculated with PVY-VN at an approximate concentration of 2 μg/ml in 10 mM phosphate buffer, pH 7.2. Then the inoculated leaf surfaces were rinsed with tap water. The plants were grown in the greenhouse at 22-30℃. Symptom development was monitored on a daily basis after inoculation.

Detection of the gene introduced in resistant transgenic tobacco plants. Polymerase chain reaction (PCR) analysis was used for the detection of the introduced gene. The same primer set for NIb cDNA cloning was used for verification of transgenic tobacco plants. 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.

RESULTS

Resistance of regenerated transformants. The putative transgenic plants, which were selected on kanamycin media (kanamycin-resistant, Km^R), were tested for their resistance to PVY-VN (vein necrosis strain). Out of 25 plants with sense-oriented gene insertion, three (2ns-1, 2ns-2 and 2ns-3) had no symptom 2 weeks after PVY-VN inoculation (Fig. 2). All the other transgenic plants and non-transformed control plants (Burley 21) showed typical vein necrosis symptoms 2 weeks after inoculation. In the further observation of symptom expression, no symptom was developed in 2ns-1 up to 10 weeks after inoculation, but in 2ns-2 and 2ns-3 mild chlorotic spot symptoms (termed as delayed symptoms) (Fig. 3) appeared from 4 weeks after inoculation.

After seed harvest, the plants were cut, and PVY-VN was inoculated on suckers grown out from the stumps. No symptom was observed in 2ns-1 up to 8 weeks after inoculation, while mild vein necrotic symptoms were developed on the suckers of the other two resistant plants (not photographed).

Resistance of T_1 transgenic plants. The three resistant transformants, 2ns-1, 2ns-2 and 2ns-3, were self-fertilized, and their progenies (T_1) were tested for resistance to PVY-VN. T_1 plants from 3 transgenic lines varied greatly in resistance incidences, comprising 92 (76.7%), 39 (32.5%) and 6 (5.0%) resistant plants out of 120 T_1 transgenic plants for 2ns-1, 2ns-2 and 2ns-3, respectively (Table 1). Only the most resistant transgenic line (2ns-1) was segregated 3:1 as Mendel's law of segregation, but the other two lines deviated greatly.

Duration of resistance in the transgenic lines were examined by expression of symptoms up to

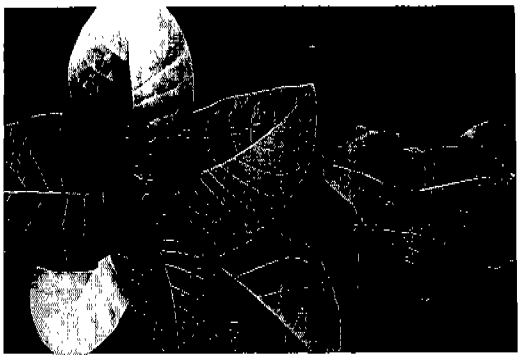

Fig. 2. Resistant (left) and susceptible (right) transgenic Burley tobacco plants, showing a healthy shape (no symptom and good growth) and PVY symptoms (vein necrosis and poor growth) 2 weeks after inoculation, respectively.

Fig. 3. Chlorotic spot symptoms appeared on the upper leaf of a resistant transgenic plant 10 weeks after inoculation.

Table 1. PVY resistance of the 1st progeny (T_1) of the transgenic plants 2 weeks after inoculation

Transgenic line tested	No. of total plants	No. of resistant plants(R)	No. of susceptible plants(S)	R:S ratio	χ^{2a}
2ns-1	120	92 (76.7)[b]	28 (23.3)	3.3 : 1	0.14
2ns-2	120	39 (32.5)	81 (67.5)	1 : 2.1	74.10
2ns-3	120	6 (5.0)	114 (95.0)	1 : 19.0	961.22

[a] χ^2 analysis for 3 : 1 segregation of resistance. $\chi^2_{0.05} = 3.84$.
[b] Numbers in parentheses are percentages to the total.

10 weeks after inoculation and on suckers grown out from tobacco stumps after seed harvest. In 2ns-1, out of 20 plants tested (with no symptom at 2 weeks after inoculation), only one plant showed symptoms at 10 weeks after inoculation, termed as delayed symptoms in this study, and 5 plants had mild chlorotic mottling symptoms on suckers (especially young suckers) (Table 2). In 2ns-2, one plant out of 7 had delayed symptoms on main leaves, and 2 plants had symptoms on suckers. In 2ns-3, only one plant out of 3 was symptomless up to 10 weeks after inoculation and on suckers.

Table 2. Duration of PVY resistance of the 1st progeny (T_1) of the transgenic plants[a]

Transgenic line tested	No. of total plants	No. of plants with delayed symptoms[a]	No. of plants with diseased suckers[b]
2ns-1	20	1 (5.0)[c]	5 (25.0)
2ns-2	7	1 (14.3)	2 (28.6)
2ns-3	3	2 (66.7)	2 (66.7)

[a] Symptom expression was delayed (symptoms of chlorosis were observed at 10 weeks after inoculation). [b] After seed harvest, plants were cut, and PVY-VN was inoculated on suckers grown out from the plant stumps. [c] Numbers in parentheses are percentages to the total.

Resistance of T_2 transgenic plants. In the line 2ns-1 of T_1, 19 plants which had no symptoms up to 10 weeks after inoculation were self-fertilized, and the progenies (T_2) were tested for PVY resistance. At two weeks after inoculation, there were variations in the level of resistance, showing

Table 3. PVY resistance of the 2nd progenies (T_2) of the transgenic plant

Plant line	No. of plants tested	Level of resistance[a] (%)		
		High resistance	Moderate resistance	Susceptibility
Burley 21	72	0	0	100
2ns-1-1[b]	137	32.9	29.9	37.2
2ns-1-2	108	39.8	36.1	24.1
2ns-1-3	142	60.5	28.9	10.6
2ns-1-4	144	68.1	22.9	9.0
2ns-1-5	143	80.4	0	19.6
2ns-1-7	144	0.0	0.0	100.0
2ns-1-9	144	67.4	9.7	22.9
2ns-1-10	144	59.7	14.6	25.7
2ns-1-11	144	57.6	21.5	20.9
2ns-1-12	144	2.0	66.7	31.3
2ns-1-13	144	36.8	36.1	27.1
2ns-1-16	144	0	2.8	97.2
2ns-1-17	108	59.3	19.4	21.3
2ns-1-18	142	12.0	49.3	38.7
2ns-1-20	71	81.7	0	18.3
Subtotal	2003	43.9	22.5	33.6
2ns-1-6[c]	144	22.9	38.9	38.2
2ns-1-8	144	22.2	38.2	39.6
2ns-1-14	108	11.1	7.4	81.5
2ns-1-15	144	0.0	0.0	100.0
2ns-1-19	108	70.3	13.0	16.7
Subtotal	648	25.3	19.5	55.2

[a] Level of resistance: high resistance; no symptom, moderate resistance; mild chlorotic spot symptoms on 1-2 lower leaves, and susceptibility; vein necrosis symptoms at 2 weeks after inoculation.
[b] Coll lines with no symptom on suckers at T_1.
[c] Coll lines with symptom on suckers at T_1.

high resistance (no symptom), moderate resistance (mild chlorotic spot symptoms on 1-2 lower leaves), and susceptibility (vein necrosis symptoms). All of the non-transformed Burley 21 tobacco plants showed severe PVY-VN symptoms (vein necrosis). In the transgenic lines with no symptom on suckers in T_1, two lines had all or almost susceptible plants, and the other 14 lines had 9.0 - 38.7 % susceptible plants (Table 3). The average percentage of resistant plants in the transgenic lines was 66.4%. In the transgenic lines with symptoms on suckers, three out of 5 lines had more than 60 % resistant plants, and the average resistance percentage was 44.8%.

Thirty or 50 T_2 plants with no symptom at 2 weeks after inoculation were selected from the 6 most resistant lines, and symptom development was examined up to the seed-harvesting time. In the 6 lines, no plant showed susceptible symptoms, and 66.7-100 % of the plants remained symptomless (Table 4). The degree of delayed symptom development varied depending on the cell lines, ranging from 0 to 33.0 %.

Table 4. Symptom development at the later stage (10 weeks) after inoculation in the initially symptomless T_2 progenies

Cell line	No. of plants observed	% plants with		
		No symptom	Delayed symptom[a]	Severe symptom
2ns-1-3	30	66.7	33.3	0.0
2ns-1-4	30	100.0	0.0	0.0
2ns-1-5	50	98.0	2.0	0.0
2ns-1-9	30	90.0	10.0	0.0
2ns-1-11	30	80.0	20.0	0.0
2ns-1-20	30	93.3	6.7	0.0

[a] Delayed symptom; development of symptoms with mild chlorotic spots to chlorotic mottling in 10 weeks after inoculation.

Verification of gene introduction in the resistant transgenic plants. Polymerase chain reaction (PCR) was used to demonstrate whether the inserted gene was present in the putative Km^R transgenic tobacco plants. Two specific primers for direct detection of 1.5 kb replicase gene were used for PCR analysis. The amplified DNA samples were then fractionated by electrophoresis on a 0.8% agarose gel. Amplified product could be detected from the transformed plants which showed no symptom 2 weeks after inoculation, but not from the non-transformed plant, and the inserted gene could be detected in resistant T_2 transgenic plants by PCR analyses (Fig. 4) as well as T_1 plants, indicating that the gene was inherited to the progenies.

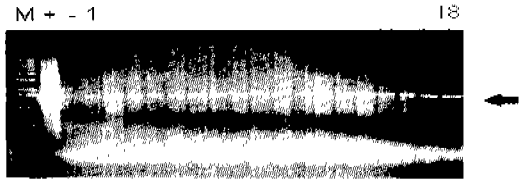

Fig. 4. Detection of the inserted cDNA of PVY-VN NIb by PCR analysis. Note the inserted cDNA was detected in all the resistant transgenic plants (T_2). (M: 1 kb ladder, +: positive control (plasmid DNA), -: negative control (a healthy untransformed plant), 1-18: resistant transgenic plants).

DISCUSSION

Out of 25 putative transgenic tobacco plants inserted with PVY-VN NIb gene (sense-oriented), only 3 appeared resistant to the PVY infection at 2 weeks after inoculation. One transgenic plant was highly resistant, showing no symptom development up to the seed harvest time, but the other 2 plants were moderately resistant to PVY-VN, showing mild symptom development during the growing periods. In the T_1 generation, much more resistant progeny plants were derived from the highly resistant T_0 plant than from the moderately resistant T_0 ones, suggesting that the PVY resistance incidences of the progenies may be related to the resistance levels of the mother plants. This also suggests that the resistance conferred by the insertion of PVY NIb cDNA may be inheritable in a way or another.

However, in the T_2 of the highly resistant

transformant (2ns-1), the average resistance incidence was similar to that of T_1, but the resistance levels of the progeny lines varied greatly, ranging from totally susceptible to highly resistant. This variation of resistance level in the posterior generation was also noted in our previous study using a flue-cured tobacco cultivar transformed by the same replicase cDNA (Park *et al.*, 1997). Superficially only one copy of T-DNA appeared to be inserted in the plant because the resistance incidences were segregated 3:1 in the T_1 as Mendel's law of segregation. On the other hand, the segregation ratio at T_2 indicates that the plants may have multiple inserts. Further experiments on the number and location of the inserted genes are needed for explaining these phenomena.

According to Baulcombe in 1996, mechanisms of pathogen-derived resistance can largely be divided into two categories, resistance with or without gene silencing. Resistance independent of gene silencing is conferred by products (coat and movement proteins and RNA or DNA) mediated by transgenes. Resistance involving gene silencing (or RNA-based homology-dependence resistance) has been established from the facts that the accumulation of transgene-mediated RNAs is not correlated with virus resistance (Lawson et al, 1990) and that resistance is also conferred by modified viral transgenes (De Haan *et al.*, 1992; Lindbo and Dougherty, 1992; Van de Vlugt *et al.*, 1992). Audy *et al.* (1994) suggests that the resistance mechanism conferred by PVY replicase may be independent of gene silencing. However, this mechanism cannot explain the decrease of resistance in T_2 plants even if the plants might have multiple copy of the transgene, because it is hardly probable that the protein production was retarded or suppressed in the T_2 generation. In an experiment of ours, on the other hand, transformants with cDNAs of the 3′ and 5′ deleted NIb sequences also showed resistance to the PVY infection as full NIb sequence (unpublished), which may reflect the gene silencing mechanism of the resistance. In this respect, the resistance conferred by PVY replicase cDNA in our experiment is more likely to be the RNA-based homology-dependence resistance.

Resistance levels varied among transgenic plants, and the initial resistance seemed to be lowered in the T_2 generation of the highly resistant transformant (2ns-1), even the transgene exists in the plant genome in our study. The viral NIb gene was detected in the highly resistant, moderately resistant, and even some susceptible transformants (data not shown). This phenomenon also support the gene silencing mechanism of resistance in which various factors such as DNA methylation, transgene copy number and the repetitiveness of the transgene insert, transgene expression level, possible production of aberrant RNAs may be involved alone or in combination. However, the exact mechanism explaining above mentioned phenomena was not revealed in this experiment. Based on our experimental, it can be concluded that the inserted gene was inherited to the progenies, but that the resistance may not be governed by gene introduction itself, but by the expression of the inserted gene related to mechanisms of resistance.

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