Journal of the Korean Society of
Tobacco Science Vol. 19, No. 1, 11~17(1997)
Printed in Republic Korea.

Resistance Characteristics of Flue-cured Tobacco Plants Transformed with cDNA of Potato Virus Y Replicase Gene

Eun Kyung Park*, Kyung-Hee Paek[1], Jin Sam You[1], Hye Sun Cho[2], Shin Woong Kang, and Young Ho Kim

Korea Ginseng and Tobacco Research Institute, Taejon 305-345, Korea,
[1]Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea
[2] Plant Cell and Molecular Biology Research Unit, KRIBB, KIST, Taejon 305-333, Korea
(Received May 19, 1977)

감자 바이러스 Y 복제유전자 cDNA로 형질전환된 황색종 담배의 저항성 특성

박은경*, 백경희[1], 유진삼[1], 조혜선[2], 강신웅, 김영호
한국인삼연초연구원, [1]고려대학교 생명공학원, [2]생명공학연구소
(1997년 5월 19일 접수)

ABSTRACT: A flue-cured tobacco variety (*Nicotiana tabacum* cv. Wisconsin) was used for plant transformation with the complementary DNA (cDNA) of potato virus Y-necrosis strain (PVY-VN) replicase gene (NIb) which was synthesized through reverse-transcription primed with oligo(dT) and polymerization using RNase H-digested template. The cDNA was cloned into plant expression vector plasmid (pMBP2), and introduced into tobacco plants by co-culturing tobacco leaf disks with *Agrobacterium tumefaciens* LBA4404 containing the plasmid before plant regeneration. Eight plants, in which the inserted cDNA fragment was detected by polymerase chain reaction (PCR), out of 70 putative transformants inserted with sense-oriented NIb cDNA showed no symptom at 3 weeks after inoculation, while the other 62 plants, and all plants with vector gene only and antisense-oriented NIb cDNA had susceptible vein-necrosis symptoms. However, only 2 of the 8 resistant plants were highly resistant, which remained symptomless up to 10 weeks after inoculation. Among the first progenies (T_1) from self-fertilized seeds of the two resistant transgenic plants, less than 10 % of T_1 plants appeared highly resistant (with no symptom), 70 % moderately resistant (with mild symptoms on 1 - 2 leaves), and about 20 % susceptible (with susceptible symptoms on 3 or more leaves) at 3 weeks after inoculation. These results suggest that the PVY resistance was inherited in the T_1 generation.

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

In Korea PVY occurs both in flue-cured and air-cured tobacco cultivars, but more severely in the air-cured (burley) tobacco. However, in some flue-cured tobacco (NC 82) areas where potato is collectively growing, tobacco plantations are devastated by PVY in recent years (Park et al., 1995; 1996),

* Corresponding author : *Korea Ginseng & Tobacco Research Institute, 302 Shinsong-dong, Yusong-ku, Taejon 305-345, Korea*
* 연락저자 : 대전광역시 유성구 신성동 302, 한국인삼연초연구원

and the disease is the most important limiting factor to tobacco cultivation in these areas.

PVY is usually controlled by controlling insect vectors using insecticide spray and/or by preventing their movement from field to field (Lucas, 1975). However, the control of aphids does not give sufficient control efficacies for PVY infection, since they can transmit the virus before the lethal effect works upon their contact with insecticides, and no cultural practices can fully block their movement. The most efficient way of controlling PVY is use of tobacco cultivars resistant to PVY.

It is difficult to develop reliable resistant tobacco cultivars in conventional breeding programs, especially in flue-cured tobacco which governs the taste of tobacco smoking mostly, not because of the lack of resistance gene sources but because of the introduction of unwanted traits together with the resistance genes in breeding. In recent years, recombinant DNA techniques have been applied to develop resistant transgenic plants mainly transformed with viral genes such as coat protein genes (CP), replicase genes, antisense RNAs, and satellite RNAs, among which CP-mediated virus resistance is most extensively studied. (Beach, 1993). By using the techniques, resistance can be established in transgenic plants with minimum alteration of characteristics of mother plants.

Replicase gene-mediated virus resistance has been little studied. In this study, transgenic tobacco plants were developed by transformation of tobacco plants with PVY-VN replicase gene, and characteristics related to the resistance of the transgenic plants to PVY-VN were examined. Also inheritance of the resistance was investigated to give information on determining the reliability of transgenic plants transformed with PVY-VN replicase gene in the practical application of the disease control.

MATERIALS AND METHODS

RNA isolation from purified PVY-VN particles. RNA was isolated from purified virus which was separated from tobacco leaves (*Nicotiana tabacum* cv. Burley 21) infected with PVY-VN by cesium chloride (38%) density gradient centrifugation. The purified virus was suspended in TNE buffer (10mM Tris-HCl, pH 7.5, 100mM NaCl, 10mM Na_2EDTA) with 4 % sodium dodecyl sulfate (SDS), and an equal volume of phenol saturated with TNE were added. The mixture was shaken intermittently on ice for 10 min and centrifuged, and the aqueous phase was recovered and re-extracted with the TNE-saturated phenol. The RNA was concentrated by ethanol precipitation with 1/10 volume of 3 M sodium acetate (pH 5.5), and dissolved in diethylpyrocarbonate (DEPC)-treated distilled water.

cDNA synthesis and cloning. The bacterial strain used for transformation and plasmid conservation is *E. coli* HB101 [*supE44 hsdS20(rB⁻mB⁻) recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl5 mtl1*]. For cDNA synthesis, reaction conditions were essentially those from the Amersham cDNA synthesis system (Amersham International plc, UK). Viral RNA (4 μg) isolated as above was reverse transcribed into cDNA primed with oligo(dT) during the first strand synthesis, and RNase-H-digested template and Klenow polymerase were used for the second strand synthesis. Reactions were quantified by the incorporation of [α-^{32}P]dCTP. The product was treated with T4 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 plasmid 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 cycles) was carried out to clone NIb 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, pMBP-2, as *Bam*HI fragment. The orientation of the inserts, antisense or sense, was confirmed by *Sma*I and *Bgl*II restriction enzyme analysis.

Nucleic acid sequencing. Nucleotide sequencing was carried out in plasmid pUC19 by the dideoxynucleotide chain termination method of Sanger et al. (1977) using Sequanase 2.0 (USB, USA). Universal M13 primers for reverse and forward

reactions were used and [α-^{35}S]dATP-labeled products were electrophoresed on 6% polyacrylamide gels containing 8 M urea.

Plant transformation and regeneration. The plasmids containing the PVY-VN NIb cDNA clone were transferred from *E. coli* HB101 into *Agrobacterium tumefaciens* strain LBA4404 by direct DNA uptake. The structure of the vector mobilized into *Agrobacterium* was verified by restriction enzyme analysis of purified *Agrobacterium* plasmid DNA. Tobacco 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 supplemented with 100 mg/l kanamycin. After root development (2 to 3 weeks), plantlets were transferred to soil and developed into the whole plant in a greenhouse. Transgenic plants were fully grown in the greenhouse and self-fertilized to produce seeds. The first progenies (T1) were grown and tested for resistance to PVY in the greenhouse.

Detection of the introduced gene in transgenic tobacco plants. PCR was applied for the analysis of the gene in transgenic plants with the same primer set for NIb cDNA cloning. 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 and 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. Tobacco plants about 3 weeks after transfer to soil (at the stage of 4-5 true leaves) were used for assays of virus infection. Two leaves from 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 or plant sap dilution (diluted 100 times with the buffer). After the inoculated leaf surfaces were rinsed with tap water, the plants were grown in the greenhouse at 22-30℃. Symptom development was observed at intervals of one week after inoculation.

Detection of viral infection was also done serologically by using direct double sandwich ELISA method described by Clark and Adams (1977). The optimum dilutions of antiserum and plant sap were always checked before testing serological reactions.

RESULTS

Analysis of PVY-VN NIb gene cloned in plant expression vector. In this experiment, the cloned NIb cDNA was recombined in the direction of sense or antisense to plant expression vector pMBP-2 with 35S double promoter to enhance the expression of the inserted gene in the plant system. In the sequencing of the gene (data not shown), the PVY-VN NIb was 1,557 nt-long expressing 57 kDa protein, and had 92% to 93% identity to those of other PVY strains.

Resistance of 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 70 plants with sense-oriented gene insertion, 8 had no symptom at 3 weeks after PVY-VN inoculation, while all the transgenic plants with only vector gene or antisense-oriented replicase gene showed susceptible symptoms (Table 1, Fig. 1). However, among the 8 resistant plants, only 2 transgenic plants (Nw08 and Nw66) remained symptomless up to 10 weeks after inoculation. In the other plants symptoms appeared from 4 weeks after inoculation.

Detection of virus in transgenic plants. The 8 transgenic plants having no PVY symptom at 3 weeks after inoculation were tested for the viral presence in the inoculated and upper leaves by bioassay and serology. Virus was detected by both inoculation test and ELISA in all the inoculated leaves, but not in the upper leaves except one plant that showed symptom on the upper leaves at 4

Table 1. Resistance of regenerated transgenic plants (T_0) to PVY infection determined by symptom expression after PVY-VN inoculation

Gene	Direction	No. plants tested	No. plants without symptoms after*	
			3 weeks	10 weeks
Vector	Sense	60	0	-
Replicase	Sense	70	8	2
	Antisense	60	0	-

* PVY-VN was inoculated on two leaves for each plant at 4 to 5 leaf stages, and symptom expression was observed visually at intervals of one week after inoculation.

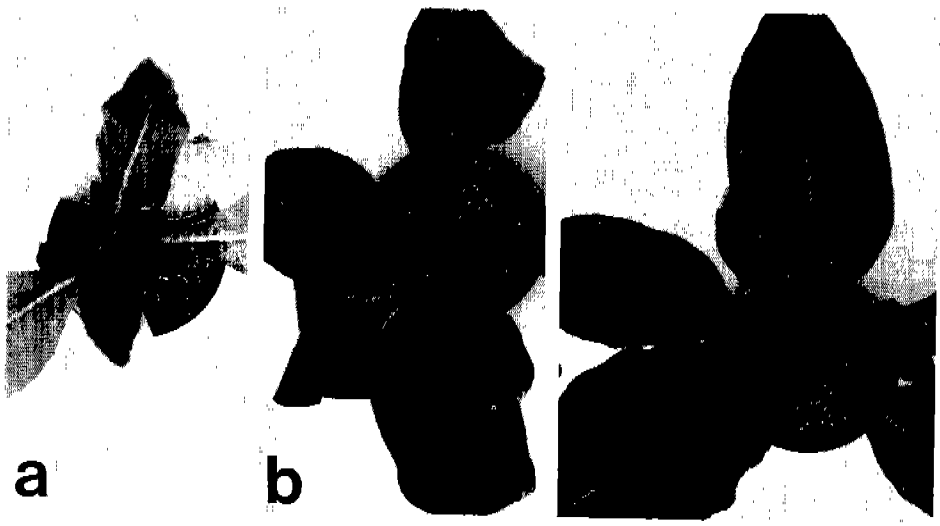

Fig. 1. Resistant (c) and susceptible (b) transformants transformed with PVY-VN NIb cDNA, and a susceptible non-transgenic plant (a). No symptom appeared on the resistant transformant, while the susceptible ones had vein-necrosis symptoms and showed poor and delayed plant growth.

weeks after inoculation (Table 2).

Verification of gene introduction. Polymerase chain reaction (PCR) was used to demonstrate whether T-DNA was present in the resistant transgenic tobacco plants. Two specific primers for direct detection of 1.5 kb replicase gene were used for PCR analyses, and the amplified DNA samples were fractionated by electrophoresis on a 0.8% agarose gel. The product amplified by PCR was detected in all of the resistant transgenic plants but not in non-transformed plants (Fig. 2).

Evaluation of resistance in T_1 progenies of the resistant lines. The 2 transgenic lines with no symptom up to 10 weeks after inoculation were self-fertilized, and progenies (T_1) were tested for resistance to PVY-VN. Three weeks after inoculation, T_1 plants from the 2 lines showed both resistant and susceptible responses. As resistant responses, 9 and 7 plants had no symptom, and 73 and 72 plants had mild symptoms on 1-2 leaves out of 102 and 103 plants in Nw08 and Nw66, respectively (Table 3). Susceptible progenies were

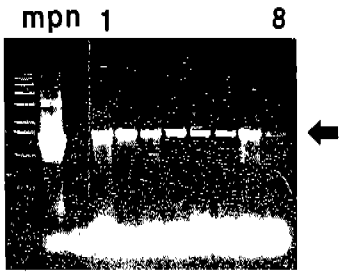

Fig. 2. Detection of PVY-VN NIb gene (1.5 kb) in transgenic plants (T_0) by PCR analysis. Lane m: 1 kb ladder, lane p: positive control (PVY-VN NIb cDNA-containing plasmid), lane n: negative control (healthy non-transformed plant), lanes 1-8: resistant transgenic plants.

about 20%. In the statistical analysis, the ratio of resistant (including moderately resistant) to susceptible plants did not deviate 3 to 1 significantly.

DISCUSSION

In our study, two transgenic lines showed a high resistance to PVY-VN, while the other 68 transgenic plants with sense-oriented PVY-VN NIb and all of the plants with the antisense-oriented gene became systemically infected at early or late stages of infection. In a preliminary study, susce- ptible transgenic plants as well as resistant ones contained the viral gene (unpublished). The viral gene was also detected in the transgenic plants which showed no symptom initially but PVY symptoms later in the

Table 2. PVY infection in inoculated and upper leaves of resistant transgenic plants (T_0)

Access transformant number	Inoculated leaves		Upper leaves	
	ELISA	Inoculation test*	ELISA	Inoculation test
Nw08	+	+	-	-
Nw40**	+	+	+	+
Nw61	+	+	-	-
Nw62	+	+	-	-
Nw63	+	+	-	-
Nw64	+	+	-	-
Nw65	+	+	-	-
Nw66	+	+	-	-

* Symptom development was observed on Burley 21 plants inoculated with leaf sap dilution (x 50) of the transgenic plants.
** Symptoms were developed at 4 weeks after inoculation.

Table 3. PVY resistance of the 1st progenies (T_1) of the transgenic plant lines determined by symptoms at 4 weeks after inoculation

Transgenic line	No. plants tested	No. of plants*			Resistant: susceptible ratio	χ^{2**}
		Highly resistant	Moderately resistant	Susceptible		
Nw08	102	9 (8.8)***	73 (71.6)	20 (19.6)	4.1 : 1	1.58
Nw66	103	7 (6.8)	72 (69.9)	24 (23.3)	3.3 : 1	0.17

* Highly resistant: no symptom, moderately resistant: mild symptoms developed on 1 or 2 leaves, susceptible: symptoms developed on 3 or more leaves in a plant.
** χ^2 analysis for 3 : 1 segregation of resistance. $\chi^2_{0.05} = 3.84$.
*** Numbers in parentheses are percentages to the total.

present study. These results suggest that the gene introduction into the plant system may not necessarily provide resistance to transgenic plants. Probably resistance expression via the inserted gene may be governed by number, direction and position of the inserted gene in the plant genome. No resistant transformants were obtained through antisense CP mediation in a previous study (unpublished) as well as antisense NIb mediation in the present study. In general, the expression of antisense RNAs has not led to high levels of virus resistance (Powell et al., 1989), although antisense RNAs should decrease viral replication in principle.

All of the resistant transgenic plants transformed with sense-oriented NIb were infected by PVY-VN in our study. However, the virus was confined in the inoculated leaves at an initial stage of infection, delaying symptom expression. These results suggest that the replicase gene-mediated resistance may not decrease the viral infectivity but limit viral multiplication in the plant system. Phenotypes of the resistance such as delayed symptom expression, inhibited systemic infection, and reduced disease severity might be related to the limited viral multiplication in the transgenic plants. Transgenic plants that express PVY CP also accumulate much less virus, following inoculation with the virus (Lawson et al., 1990).

In case of tobacco mosaic virus (TMV), probable mechanisms of resistance may be involved with cross protection which renders resistance rather by gene expression products (coat protein and replicase fusion protein products) (Powell-Abel et al., 1986; Sanders et al., 1992, Donson et al., 1993). Also in replicase-mediated resistance against PVY, the resistance mechanism was suggested as protein based (Audy et al., 1994), although there are many examples of transgenic plants transformed with replicase genes that show RNA-mediated resistance (Baulcombe, 1996). However, in CP-mediated resistance, there is no correlation between CP levels in transgenic plants and PVY resistance. More studies on PVY resistance mechanisms of transgenic plants transformed with replicase gene are needed.

Immune and resistant phenotypes were expressed in transgenic tobacco plants transformed with a potyvirus CP nucleotide sequence (Lindbo and Dougherty, 1992). In our study, no immune plants were obtained by transforming tobacco plants with NIb cDNA. In the T_1 progeny of the highly resistant transformants of the flue-cured tobacco, resistance (including moderate resistance) was inherited, segregating about 3:1, which was corresponding to the Mendel's law of segregation. This suggests that the inserted gene might be stable during the sexual reproduction including gamete formation and chromosome paring. However, compared to the transformants (T_0) which showed no symptom up to 10 weeks after inoculation, most of resistant T_1 plants showed mild symptoms on 1-2 leaves, indicating that the resistance levels might be diversified in the T_1 generation.

요 약

감자 바이러스 Y 피저계통 (PVY-VN) 복제유전자 cDNA로 형질전환된 황색종 담배 (*Nicotiana tabacum* cv. Wisconsin)의 PVY에 대한 저항성 특성을 조사하였다. 순수분리한 바이러스에서 RNA를 분리한 후 역전사 (reverse transcription)와 중합 (polymerization)으로 복제유전자 cDNA를 합성하였다. 이 cDNA는 식물발현벡터에 클로닝한 후 *Agrobacterium tumefaciens* LBA 4404로 식물체에 도입되었다. 정방향으로 유전자가 도입된 형질전환체 식물체 70주 중 8주가 PVY 접종 3 주후 병징 발현이 되지 않아 저항성으로 나타났으나, 역방향으로 삽입된 유전자를 가진 형질전환체와 벡터 유전자만 삽입된 식물체는 모두 감수성을 나타내었다. 이들 저항성 담배 식물체에서는 삽입한 유전자가 PCR 분석에 의해 확인되었다. 이들 식물체중 2 주가 접종 10주 후에도 병징을 보이지 않아 고도의 저항성을 보였으며, 자가수정 후 T_1 세대에서의 저항성주율이 3:1로 분리되어 PVY 저항성이 다음 세대로 유전됨이 확인되었다. 그러나 T_1 세대에서는 T_0 세대보다 저항성정도의 변이가 증가하였다.

REFERENCES

1. Audy, P., P. Palukaitis, S. A. Slack, and M. Zaitlin (1994) Replicase-mediated resistance to potato virus Y in transgenic plants. *Mol. Plant Microbe Interact.* 7: 15-22.
2. Baulcombe, D. C. (1996) Mechanisms of pathogenderived resistance to viruses in transgenic

plants. *Plant Cell* 8 : 1833-1844.

3. Beachy, R. N. (1993) Virus resistance through expression of coat protein genes. In: Biotechnology in Plant Disease Control, ed by I. Chet, p. 89-104. A John Wiley & Sons Inc. Publication, New York.
4. Clark, M. F. and A. N. Adams (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34: 475-483.
5. Donson, J., C. M. Kearney, T. H. Turpen, I. A. Khan, G. Kurath, A. M. Turpen, G. E. Jones, W. O. Dawson, and D. J. Lewandowski (1993) Broad resistance to tobamoviruses is mediated by a modified tobacco mosaic virus replicase transgene. *Mol. Plant-Microbe Interact.* 6: 635-642.
6. Horsch, R. B., J. E. Fry, N. L. Hoffman, D. Eichholtz, S. G. Rogers, and R. T. Fraley (1985) A simple and general method for transferring genes into plants. *Science* 227: 1229-1231.
7. Lawson, C., W. K. Kaniewski, L. Haley, R. Rozman, C. Newell, P. Sanders, and N. E. Tumer (1990) Engineering resistance to mixed virus infection in a commercial potato cultivar: Resistance to potato virus X and potato virus Y in transgenic Russet Burbank. *Bio/Technology* 8: 127-134.
8. Lindbo, J. A. and G. W. Dougherty (1992) Pathogen-derived resistance to a potyvirus: Immune and resistance phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence. *Mol. Plant-Micro. Interact.* 5: 144-153.
9. Ling, K., S. Namba, C. Gonsalves, J. L. Slightom, and D. Gonsalves (1991) Protection against detrimental effects of potyvirus infection in transgenic tobacco plants expressing the papaya ringspot virus coat protein gene. *Bio/Technol.* 9: 752-758.
10. Lucas, G. B. (1975) Diseases of Tobacco, Third Edition, pp. 621 Biological Consultants Associates, Raleigh, North Carolina.
11. Murashige, T., and F. Skoog. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant* 15: 473-497.
12. Park, E. K., Y. H. Kim, S. Y. Chae, S. W. Kang, S. S. Kim, and W. H. Yeo (1995) Management of viral diseases and biological control of pathogen and insect pests. Tobacco Research Report (Tobacco Breeding and Environment), p. 69 - 151. Korea Ginseng and Tobacco Research Institute.
13. Park, E. K., S. S. Kim, Y. H. Kim, S. Y. Chae, and S .W. Kang (1996). Studies on biological and ecological control of tobacco viruses and quality-degrading agents. Tobacco Research Report (Tobacco Breeding and Environment), p. 173 - 244. Korea Ginseng and Tobacco Research Institute.
14. Powell, P. A., D. M. Stark, P. R. Sanders, and R. N. Beach (1989) Protection against tobacco mosaic virus in transgenic plants that express tobacco mosaic virus antisense RNA. *Proc. Natl. Acad. Sci. USA* 86: 6949-6952.
15. Powell-Abel, P., R. S. Nelson, B. De, N. Hoffmann, S. G. Rogers, R. T. Fraley, and R. N. Beachy (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* 232: 738-743.
16. Sanger, F., S. Nicker, and A. R. Coulson (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acd. Sci. USA* 74: 4463-4467.
17. Sanders, P. R., W. K. Kaniewski, L. Haley, B. LaVallee, X. Delannay, and N. E. Tumer (1992) Field trials of transgenic tomatoes expressing the tobacco mosaic or tomato mosaic virus coat proteins. *Phytopathology* 82: 683-690.