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Stable Expression of TMV Resistance and Responses to Major Tobacco Diseases in the Fifth Generation of TMV CP Transgenic Tobacco

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ABSTRACT : TMV resistant lines (TRLs) originated from the R_0 plant of *Nicotiana tabacum* cv. NC82 transformed with TMV coat protein cDNA which initially showed delayed disease symptom were selected for increased resistance in each subsequent generation. The result of field experiment of the transgenic tobacco lines in the fifth generation for TMV resistance and their response to other tobacco diseases (black shank, bacterial wilt, and powdery mildew) is described in this report. When fifteen TRLs of the fifth generation were tested for TMV resistance by mechanically inoculating the individual plants, over 95 percent of the plants of 6 lines showed complete resistance even 8 weeks after the inoculation. Average frequency of the resistant plants in TRLs of the fifth generation 8 weeks after the inoculation was 87%. Stable insertion and expression of TMV coat protein cDNA in the fifth generation of the transgenic tobacco plant were confirmed by PCR and immunoblot hybridization, respectively. All TRLs were resistant to the black shank but were susceptible to the bacterial wilt disease and the powdery mildew to the same degree as non-transgenic NC82 was. Therefore, it was indicated that the phenotypes related at least to disease resistance were not changed in the transgenic tobacco.

***Key words* : TMV CP cDNA, TMV resistant tobacco plant, transformation.**

Recombinant DNA technique is expected to bring about a great progress in the development of new plant varieties showing excellent pest and disease resistances as well as high quality. Developing transgenic plants using a foreign DNA from plant virus has been routinely performed with special interest in many laboratories since Powell Abel *et al.* (1989) reported delay phenomenon of disease development in transgenic tobacco plants that expressed the tobacco mosaic virus (TMV) coat protein gene. Cuozzo *et al.* (1988) reported that tobacco plants transformed with cucumber mosaic virus (CMV) coat protein gene or antisense RNA were protected from the virus infection. Resistances to viral infection in tobacco plant induced by transgenes such as cDNA of grapevine fanleaf nepovirus (GFNV) and potato virus Y (PVY) have been reported by Bardonnet *et al.* (1994) and Smith *et al*(1994). Transgenes other than coat protein cDNA could also result in resistance to the

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infection of more than two viruses. Maiti *et al.* (1993) reported that tobacco plant transformed with tobacco vein mottling virus (TVMV) proteinase cDNA was resistant not only to the virus but also to other viruses such as tobacco etch virus (TEV) and potato virus Y (PVY). Donson *et al.* (1993) reported that TMV replicase cDNA transformed tobacco plant was resistant to the other viruses such as tomato mosaic virus (ToMV), tobacco mild green mosaic viurs (TMGMV), and ribgrass mosaic virus (RMV). Many evidences suggest that the expression of the transgene coding for viral coat protein plays a major role in the mechanism of disease resistance by uncoating the entering virus (Wu *et al.* 1990), by inhibiting the replication of the virus due to over-accumulation of the coat protein (Clark *et al.* 1990), or by either competing with or detering the virus in transcription of RNA (Smith *et al.* 1994). However, the biological basis for those phenomena has not been completely understood. Therefore, our effort has been focused on the breeding of a virginia type tobacco variety to be introduced in the field with resistance to TMV expressed by a CP cDNA transgene from the virus. In this paper, we report the stable expression of the TMV resistance during field cultivation and the responses to other tobacco diseases including black shank, granville wilt, and powdery mildew in the 5th generation of TMV-CP cDNA transformed tobacco.

MATERIALS AND METHODS

Selection for the TMV resistance of the transgenic tobacco plant. A truncated TMV coat protein of cDNA (5708-6411) was inserted into the plasmid pBI121 plant expression vector and the recombinant plasmid (pSK101) was transformed to *Agrobacterium tumefaciens* LBA4404 which was used for the transformation of *N. tabacum* cv. NC82 (Lee *et al.* 1993). The insertion of cDNA in the regenerated transgenic tobacco plant genome was confirmed by Southern blot hybridization and the TMV coat protein expression of the inserted cDNA in the regenerated tobacco plants was confirmed by immunoblot hybridization (Lee *et al.*, 1996).

The transgenic tobacco (NC82) plants were self-fertilized in order to harvest seeds for each generation. Seedlings were grown in a glass house. Usually at 2-4 leaf stage, plants were transplanted to the field and inoculated with TMV at 3-5 leaf stage. The viral inoculum (TMV common strain isolated from Korea) was prepared from TMV-infected tobacco plant tissue and diluted 1:6 (w/v) in 0.01 M potassium phosphate buffer (pH 7.2). TMV resistance was examined every two weeks after the inocualtion and percentages of TMV resistance plants out of the total plants were counted.

Genetic analysis of transgenic tobacco plant. Tobacco chromosomal DNA was isolated from 1g of leaf tissue grown in the field according to the method of Lee *et al.* (1996). Two specific oligo-nucleotides derived from TMV CP cDNA were used as primers for PCR. One (5'-TCTTA CAGTATCACTACTCCATCTCAG-3') is located at the position from 5715 to 5741 of the TMV CP cDNA and the other (5'-ATAAGATCCGGTTCC TCTGATCAATTC-3') at the position from 6105 to 6131. PCR was carried out in 20 μl containing 50mM Tris (pH 9.0),. 40mM KCl, 1.5mM $MgCl_2$, 50 μg/ml BSA, 0.001% gelatin, 250 μM each of dATP, dCTP, dTTP and dGTP, 1 unit of Taq polymerase (Korea Biotech Inc.) and 10 pM of each oligonucleotide primer. PCR was performed in a Single Block System(Ericomp) for 35 cycles, with each cycle consisting of 94°C for 1 min to denature the template, 55°C for 1 min for primer annealing, and 72°C for 1.5 min for polymerization. At the end of 35 cycles, samples were incubated for 3 min at 72°C and kept at 4°C prior to gel analysis.

Response to other tobacco diseases. Black shank resistance : *Phytophthora nicotianae* var. *nicotianae* cultured for 12 days on PDA (potato dextrose agar) medium was diluted to 200ml of D.W. per plate and was used to inoculate the young transgenic plants transplanted in pots by soaking the wounded root tip in the diluted fungal suspension and transplanted to pot soil.

Bacterial wilt resistance : *Pseudomonas solanace-*

arum cultured for 48 hours on NA (nutrient agar) medium was diluted with sterile water to make an apropriate bacterial suspension(70% transmittance at 600nm). The bacterial suspension was inoculated into the stem of a sucker of young plant trasplanted in the pot using a capillary tube.

Powdery mildew resistance : Natural infection ratio of the transgenic plants in the field was examined at the harvesting stage.

RESULTS AND DISCUSSION

TMV resistance of transgenic tobacco plants in the fifth generation . A truncated TMV coat protein cDNA was transformed to *N. tabacum* cv. NC82. Regenerated transgenic tobacco plants showed delayed and mild symptom expression (chlorotic local lesion) by TMV infection (Lee *et al.* 1993). In the second generation the percentage of resistant plants was 30%, and the percentages of resistant plant were increased furthermore in the third generation (Park *et al.*, 1996). In the fourth generation, six cell lines were selected from 37 cell lines. They showed over 90% of resistant plants including delay type plants, 8 weeks after the TMV inoculation (Park, 1996). Fifteen plants were selected from the 6 cell lines and investigated for the resistance to TMV in the fifth generation. Percentages of TMV resistant plants from 1,520 progenies of 15 cell lines are shown in Table 1. Whereas non-transgenic NC82 plants showed severe mosaic symptoms, most of the transgenic plants remained symptomless at 8 weeks after artificial inoculation(Fig. 1) and no further symptom development was observed up to harvesting time. Their morphological characteristics were completely same as those of non-transgenic healthy NC82 plants.

Table 1. Expression of TMV resistance in 15 cell lines of transgenic tobacco plants at the fifth generation in the natural field conditions

Transgenic cell line	No. of plants tested	% Resistance to TMV infection in weeks[a]			
		2	4	6	8
NC82	100	0			
R40825	96	100	91	91	74
R40834	94	100	97	97	95
R41228	96	100	97	97	86
R41229	96	100	93	94	82
R41233	98	100	88	88	75
R41416	100	100	100	98	95
R41439	101	100	100	100	100
R42116	102	100	100	100	99
R42118	102	100	100	99	99
R42138	101	100	98	98	94
R42514	105	100	100	100	95
R42530	106	100	99	99	93
R42536	107	100	100	100	85
R43314	107	100	92	92	61
R43325	109	100	100	99	65

[a]Percentage of TMV resistant tobacco plants per observed plants.

Fig. 1. Transgenic tobacco plants of the fifth generation in the natural field conditions. A: non-transgenic tobacco plant in 8 weeks after inoculation. B: transgenic tobacco plants(R43325) inoculated with TMV.

The mean percentage of TMV resistant plants in the fifth generation was 86.5% at 8 weeks after TMV inoculation which was much higher than that of the fourth generation, 70%. During the period, from two to six weeks after transplanting, when tobacco plants in the field condition are mostly subject to the viral infection, the percentage of TMV resistant plants was over 97%(Table 1).

From the 15 cell lines of the fifth generation of transgenic tobacco plants, percentage of TMV resistant plants of 8 cell lines (0834, 1416, 1439, 2116, 2118, 2138, 2514, 2530) were over 90% at 8 weeks after inoculation and those of 6 cell lines (0834, 1416, 1439, 2116, 2118, 2514) were over 95% and these 6 cell lines were selected as highly TMV-resistant cell lines(Table 1). Those highly TMV resistant plant lines with good morphological phenotype(719 plants) were self-fertilized to harvest seeds.

Genetic analysis of transgenic tobacco plant. Genomic PCR was performed to see whether TMV coat protein cDNA was inserted in the fifth generation of transgenic tobacco plants. As shown in Fig. 2, amplified 417bps PCR product from the TMV coat protein cDNA was shown in both plant expression vector pSK101(Fig. 2, lane 2) and all the transgenic tobacco plants (Fig. 2, lanes 4-13)

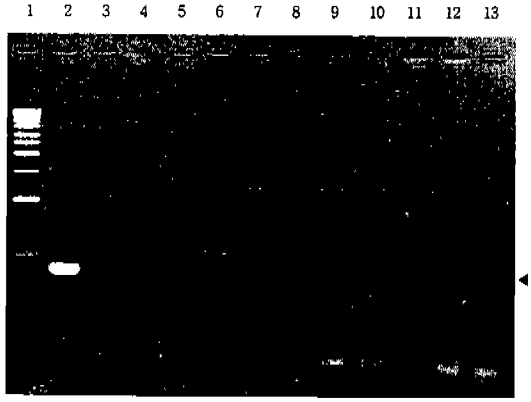

Fig. 2. Genomic PCR of transgenic tobacco plants in the fifth generation. Lane 1: 1 kb ladder, 2: pSK101 recombinant plasmid, 3: non-transgenic NC82, 4-13: transgenic NC82 of the fifth generation. The arrow indicates approximately 417 bps of amplified TMV CP cDNA product.

but not in non-transgenic tobacco plant(Fig. 2, lane 3). Expression of the transformed TMV CP cDNA in the fifth generation plants was confirmed by an immunoblot hybridization test(data not shown)

Table 2. Disease resistances of the transgenic tobacco plants

	Percentages of resistant tobacco plants to			
	TMV[a]	Black shank[b]	Powdery mildew[c]	Bacterial wilt[d]
Transformants[f]	86.5	93.9	28.4	23.0
Normal NC82	0	89.5	25.5	27.4

[a] Percentage of TMV resistant tobacco plants per examined plants in eight weeks after inoculation.

[b] Percentage of black shank resistant tobacco plants per examined plants in three weeks after inoculation.

[c] Percentage of powdery mildew resistant plants per examined total plants in the field.

[d] Disease index $= \frac{\sum (\text{severity}^{e} \times \text{No. of plants})}{\text{No. of plants} \times 5} \times 100$

[e] Disease severity: 0; no symptom NC82 on a stalk and leves, 1; symptoms on a stalk and leaves less than 1/3 in plant height, 3; symptoms on a stalk and leaves more than 1/3 and less than 2/3 in plant height, 5; symptoms on a stalk and leaves more than 2/3 in plant height at five weeks after inoculation.

[f] The fifth generation of transgenic tobacco plants.

Resistance of the transgenic tobacco to other plant diseases. Black shank resistance : *Phytophthora nicotianae* var. *Nicotianae* was inoculated to 843 plants from the 15 cell lines of the fifth generation transgenic tobacco and infected tobacco plants were examined at 3 weeks after inoculation. Percentage of trans- genic tobacco plants resistant to black shank was 82-100%. The mean percentatge of transgenic plant was 93.9% and that of non-transgenic NC82 plants was 89.5%(Table 2). The resistance of NC82 was not affected by the transformation.

Bacterial wilt resistance : *Pseudomonas solanacearum* was inoculated to 471 plants of the 15 cell lines of the fifth generation of transgenic tobacco plants and infected tobacco plants were examined at 5 weeks after inoculation. Mean disease index of the transgenic tobacco plants to bacterial wilt was 23.0 and that of non-transgenic tobacco plant was 27.4(Table 2). Both the trans-

genic and non-transgenic tobacco(NC82) were susceptible to bacterial wilt.

Powdery mildew : Incidences of natural infection by the fungus(powdery mildew), *Erysiphae cichoracearum*, in the 15 transgenic cell lines at the fifth generation was scored in the field conditions. Powdery mildew infection ratio of transgenic tobacco plants was in the wide range of 41-95% and the mean infection ratio was 71.6%(Table 2) while that of non-transgenic tobacco was 74.5%. There was no significant difference in the susceptibility which might be affected by the introduction of foreign gene between transgenic and non-transgenic tobacco plants.

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