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The technics of breeding a new transgenic tobacco cultivar resistant to mosaic diseases and its extention

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Summary This paper reports a new transgenic tobacco line NC89 resistant to both tobacco mosaic virus (TMV)and cucumber mosaic virus(CMV). It deals with the details of field experiments of the whole process of segregation and selection of the progenies of transgenic plant population. By using the gene transfer technics,some transgenic plants resistant to mosaic disease from a flue-cured tobacco cultivar NC89 were obtained in green house in 1989. Thereafter,through field inoculation and selection from the 2nd and 3rd generations,a homozygous line resistant to both viruses and with good qualities was bred out. The inheretant stability of the resistance of the new line was confirmed through the 4th to 6th generations. In 1992,the transgenic resistant line was planted about 7000 hectares in Henan province. The relative effect of controlling mosaic diseases of tobacco was 50－70%. The out-put value increased 30－50% per unit area. This is a new example of growing a transgenic virus-resistant plant in large scale in China.

Key words gene-transfer,tobacco mosaic virus,cucumber mosaic virus,new transgenictant line of tobacco

Introduction

Tobacco mosaic disease is one of the most serious diseases of tobacco in China. So far,there is no effective mothod that is available to control the disease.

Coat protein (cp) gene,satellite RNA gene and some replicase sequences have been applied in the breeding of transgenic resistant plants since mid-1980's. Because there are mainly two kinds of viruses (TMV and CWV) infecting the tobacco plants in North China,a plasmid vector with both cp genes of TMV and CMV was constructed by utilizing the CaMV 35s promoter and the rbcs transcription terminating signal. By using the bacteria of Agrobacterium tumefaciens,the constructed plasmid was introduced into the plants of a dominant flue-cured tobacco cultivar NC89,as a result,several transgenic plants with dual resistance to TMV and CMV were obtained in the green house in 1989. This is the most recently successful endeavor to accelerate the process of breeding a new cultivar by using the gene transfer technics combined with conventional breeding method. This is also the first example of the application of transgenic plants in large scale production in China.

Materials and methods

Segregation and selection of the 2nd generation of transgenic tobacco plants

Three transgenic tobacco seedlings with dual resistance to CMV and TMV were obtained. These plants were resistant to the disease in 50 and 60 days respectively after inoculation in green house.

Field experiment with the above transgenic tobacco plants was conducted during Sept. 1989 to Feb. 1990 at the suburb of Sanya city, Hainan province. The second generation of the transgenic plants were transplanted into the field plots with 100—120 plants respectively. The plants were inoculated with 0.5μg/ml of purified TMV or CMV (prepared from frozen diseased tobacco leaves, diluted 80 times (w/v), corresponding to 20μg/ml of purified CMV) in the fields.

The selection and breeding of homozygous lines (the 3rd generation) of the transgenic plants

84 plants with dual resistance selected from the 2nd generation were planted in the experimental field during Feb. to Sept. 1990 in Xinzheng county, Henan province. Each material was planted in one row in the TMV and CMV disease nurseries respectively. Each row consisted of 30 plants. A check row was established at an interval of nine rows. The inoculation method was the same as in the 2nd generation selection procedure.

Field test of the resistance of the transgenic tobacco plants (4th generation) to the disease by TMV and CMV inoculation and the production of original seed

Four homozygote lines with dual resistance were selected from the 3rd generation. The production of the original seed was conducted in Sanya, Hainan province, in Sept, 1990 to Feb., 1991. Disease nurseries were established to test the resistance of the selected lines by inoculation under field conditions. Thirty plants of each line were inoculated in the disease nursery. The plants were inoculated jointly with TMV (conc. 0.15μg/ml) and CMV (frozen diseased leaves powder, diluted 250 times). Disease incidence was surveyed every five days after inoculation.

Regional tests of the transgenic tobacco plants in Henan province (5th generation) and the analysis of the qualities of the transgenic flue-cured tobacco cultivar

Eleven comparision experiments were conducted in different locations in Henan province in 1991 to compare the difference between resistances of transgenic lines and that of the check, and to examine chemical ingredients of the transgenic cultivar in different areas. Data of some major agronomic characteristics were also collected. The transgenic lines and check cultivar were harvested and cured seperately to estimate tobacco yield, average price of cured tobacco leaves, ratio of best rank and middle rank tobacco leaves etc. Flue-cured totacco leaves were collected from different locations, 1 kg of each sample was used to evaluate the smoking properties and to analyse the internal chemical ingredients.

Results

Segregation and selection of the 2nd generation

The disease incidence data are presented in Table 1. These data indicate that the percentage of diseased plants of the transgenic plants (2nd generation) is obviously lower than that of the check cultivar NC89. The segregation ratio of the 2nd generation progeny population is not completely coincident with the theoretical single gene inheritance value (3:1). Therefore, we consider that this kind of resistance is relative resistance rather than a typical sin-

gle gene controlled qualitative inheritance. After tobacco seed maturity, more than 200 plants with high resistance and good agronomic characteristics were harvested.

Table 1. The segregation and field disease development of the 2nd generation of the transgenic plants population

Row No.	Days after inoculation			
	17	31	50	60
1 N/8—S—6	8.3	12.5	25.0	25.0
2 N/17	20.9	34.6	43.2	43.2
3 N/—1	13.7	28.6	42.9	45.1
CMV(ck)	44.7	70.6	88.2	89.4

The selection and breeding of homozygous line

1 Disease intensity of each plant-row in TMV nursery (Table 2). The percentage of diseased plants in the check plot was up to 86.7%, while that of the transgenic plants of line 3-134 was zero, and that of the line 3-64 and 3-56 were only 20%, these three lines exhibited good resistance to the disease. Therefore, these lines can be regarded as resistant to both TMV and CMV. By means of field inoculation and anti-kanamycin germinating test and Western-Blot analysis, these lines, including line 3-56, 3-64, 3-134, were homozygote, which were further proved by their resistance expressed in CMV nursery.

Table 2. Disease development in the TMV disease nursery of the 3rd generation

Row No. of transgenics progenies	Days after inoculation				
	10	21	30	45	60
3-64	0	6.0	6.0	16.6	20.0
3-134	0	0	0	0	0
3-56	0	3	10.0	20.0	20.0
ck	23.3	33.3	36.6	83.3	86.7

2. Studies on the ratio of heterozygote to homozygote in the disease nurseries The disease survey showed that the ratio of resistant to susceptible rows was conformed with the theoretical ratio after inoculation of 20 days(1:2). But the percentage of diseased plants varied greatly in 30 days after inoculation. Therefore, we confirm that the resisrance of transgenic plants is a relative resistance rather than a typical single gene qualitative inheritance (Table 3).

Table 3. Ratio of homozygote to heterozygote of the 3rd gereration plant row nursery (TMV disease nursery)

No. of transgenic plants of the 1st generation	No. of the segregating transgenic plants of the 2nd generation	Total number of rows in the plant-row nursery of the 3rd generation	Number of homozygotic rows after segregation in 30 days after inoculation					
			Calculated value	Observed value	Deviation	Square of deviation	χ^2 value	Ration with the Heterozygotic rows
N/8-s-6	1	13	4.3	3	−1.3	1.69	0.587 *	3.3:1
N/8-1	3	34	11.3	11	0.3	0.09	0.01 *	2.1:1
SD8-s-11	12	28	9.3	9	−0.3	0.09	0.02 *	2.1:1
Total		75	25	23	2	4	0.24 *	2.3:1

Table 4. Percentage of diseased plants in the disease nursery of the homozygote progeny(4th generation) of transgenic resistant tobacco line

Row No.	Days after inoculation					
	10	20	30	40	50	60
3-56	0	0	0	13.3	16.6	16.6
3-64	0	0	6.7	6.7	10.0	16.6
3-124	0	0	3.3	16.6	16.6	20.0
3-134	0	0	0	0	0	0
ck	5.0	13.3	38.3	75.0	78.3	100

Field test of the resistance of the transgenic tobacco plants

Data of disease incidence in each tested lines were presented in Table 4. There were significant difference between the disease incidence of the transgenic lines with dual resistance and that of the check cultivar at different period after inoculation. The resistance of line N/8-1-134 was the best, with no disease development occuring in 60 days after inoculation. Disease incidence of other lines all kept a low disease index level. The result revealed that the resistance of the selected homozygotes from the 4th generaton in the disease nursery was reliable. It could be used as new resistant tobacco cultivar in production.

Regional Tests and Quality Analysis of the Transgenic Flue-cured Tobacco Cultivar

1. Resistance of the transgenic and check cultivars under different field conditions

Disease survey was carried out several times in each trial site during tobacco growing season. In 1991, a slight-epidemic year, the resistance of the transgenic tobacco plants was not fully expressed. Nevertheless, the disease incidence of transgenic lines was still obviously lower than that of the check cultivar (NC89).

2. Agronomic characteristics, yield, quality and internal chemical ingredients analysis

Some major agronomic characteristics were investigated after flowering in different locations. The result showed that there was no difference between the surveyed items of the transgenic resistant cultivar and those of the check cultivar (NC89), but plant population in field of the transgenic cultivar were much stronger and uniform. Samples of Flue-cured tobacco leaves were sent to China Standardized Tobacco Quality Monitering and Test Centre to analyse their internal chemical ingredients. The result showed that there were no difference between the ingredients of the transgenic and the check cultivars, but the content of total nicotine of the transgenic cultivar showed a tendency of improvement.

Table 5. The agronomic characteristics, yield, qualities and out put-value per unit area, results of internal chemical ingredients analysis of cured tobacco leaves samples produced in different locations

Locations	Cultivar	Major agronomic characteristics, yield, qualities, internal chemical ingredients									
		Plant height (cm)	Number of leaves	Size of middle leaves (cm)		Yield (kg/mu)	Best rank of cured tobacco leaves (%)	Middle rank (%)	Total sugar (%)	Total nicotine (%)	Total nitrogen (%)
				Length	Width						
Yexian	Transgenic	96.2	19.8	67.8	27.8	143.1	21.0	69.7	11.30	4.72	2.33
	ck	90.8	20.2	64.6	25.6	148.8	15.9	70.0	10.74	4.04	2.27
Yanling	Transgenic	112.7	26.4	60.8	30.0	—	—	—	18.94	1.64	2.78
	ck	108.3	24.2	55.3	26.8	155.3	10.6	78.0	18.76	1.93	2.54
Wuyang	Transgenic	112.4	25.4	54.6	27.2	162.9	12.2	77.4	16.94	1.90	3.19
	ck	108.3	24.2	55.3	26.8	155.3	10.6	78.0	18.76	1.93	2.54
Luyi	Transgenic	87.0	21.0	—	23.0	217.7	36.0	49.0	—	—	—
	ck	85.0	21.0	—	27.0	198.5	34.0	52.0	—	—	—

3. Smoking evaluation of the flue-cured tobacco leaf samples

Some smoking characteristics such as aromatic quantity, smoking strength, ash color, etc. were tested. The result revealed that there was no difference between the tested characteristics of transgenic and that of the check cultivars.

Extension of the transgenic resistant tobacco cultivar

The sixth generation of transgenic tobacco cultivar was planted in a large areas, about 7000ha in the middle parts of Henan province in 1992.

1. Resistance in transgenic tobacco plants under field conditions

Disease survey (Table 6) was conducted at a large scale during the period from late-June to mid-July, 1992. It showed that plants from transgenic homozygote tobacco lines were highly resistant to the disease either in the typical comparison experiment fields or in large areas of fields, moreover, the difference between the resistance of trsansgenic cultivar and that of check cultivar was significant, especially in those areas with high disease incidence.

There were 10 typical comparison fields, in which the relative effect of controlling tobacco mosaic disease was 54.4—95.2%, with an average of 73.5%. Large scale disease survey was conducted in four counties, including 68 villages, 314 fields. The sampling area covered 383.8ha, in which the relative average controlling effect was 64.1%, showing the high resistance level of the transgenic tobacco plants in the epidemic year.

Table 6. Field resistance expressions of homozygote progeny (sixth generation) of the transgenic resistant line

Locations	Susceptible cultivar or resistant line	Number of villages	Number of tobacco fields	Diseased plants (%)	Disease index (%)	Relative effects of controlling disease
Yuzhou	Transgenic	16	83	33.2	9.47	41.2
	ck	8	43	49.7	16.14	
Changge	Transgenic	1	21	5.1	3.09	88.7
	ck	1	20	36.9	27.36	
Weidu	Transgenic	6	24	7.5	5.57	77.1
	ck	9	33	33.4	24.37	
Yanling	Transgenic	14	45	7.2	3.31	52.5
	ck	13	45	14.9	6.97	
Total or average		68	314	13:33	5.5:18.7	64.9

2. The economic benefit of transgenic tobacco cultivar

Data of yield per *mu* (1/15ha), average price, ratio of best-rank and mid-rank tobacco leaves were collected after harvest. The value of all the above items were improved due to the application of transgenic resistant tobacco cultivar in fields, resulting in significant economic benefits. Out-put value per *mu* was increased by 122—273 yuan(RMB), with an average of 182 yuan(RMB). Thus, the total increased value of the total area 7000 ha in which the transgenic resistant cultivar was planted was 19110 thousand yuan(RMB) in 1992.

Conclusion and discussion

By means of gene transfer technics, some transgenic tobacco plants resistant to TMV and

CMV from a flue-cured tobacco cultivar NC89 were obtained in green house in 1989, thereafter, through field segregation and selection from the 2nd and 3rd generations, several homozygous lines resistant to both viruses were obtained, the inheritance stability of those selected lines was confirmed through the 4th and 5th generation field experiments, ultimately, a new transgenic resistant tobacco cultivar was bred out, and it was named as "transgenic NC89".

During the process of segregation in the 2nd generation and the selection of homozygous line in the 3rd generation, it was found that the inheritance of the transgenic plants was not a typical single-gene controlled qualitative inheritance but a relative resistance inheritance.

The newly bred transgenic tobacco cultivar showed high resistance against tobacco mosaic disease in the epidemic year of 1992, the relative effect of controlling the disease being 50—70%.

References

Fang Rongxiang et al. (1990) Genetically engineered tobacco plants with dual resistance to TMV and CMV. *Science Reports*, 17: 1358—1359 (in Chinese)

Powell P, Beach R et al. (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science*, 232: 738—743

Tian Yingchuan et al. (1990) TMV cp-mediated transgenic tobacco plant and its resistance against. TMV. *Science in China*, 8: 822—831 (in Chinese)

Wu Shixiuan et al. (1989) Transgenic tobacco plants resistant to CMV constructed by the complementary satellite DNA singelbody. Science in China, 9: 948—956 (in china)

Ye Yin et al. (1992) Advances in plant gene engineering against viruses and its prospect. *Information on Microbiology Science and Technics*, 1: 1—14