JARQ 27, 126-136 (1993)

Evaluation of the Impact of the Release of Transgenic Tomato Plants with TMV Resistance on the Environment

Research Group for the Evaluation of the Impact of the Release of Transgenic Tomato Plants

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Abstract

Studies on the evaluation of the impact of the release of the tomato plants with introduced gene of TMV resistance on the environment were carried out from January, 1989, to January, 1992, mainly at the National Institute of Agro-Environmental Sciences (NIAES) in collaboration with the National Institute of Agrobiological Resources (NIAR) and the National Agriculture Research Center (NARC). Experiments were carried out according to the guidelines enacted by the Science and Technology Agency and the Ministry of Agriculture, Forestry and Fisheries. The following characteristics were compared between the original plants and transgenic plants: (1) growth characteristics such as plant height and vigor, (2) pollen dispersion based on fruit set on emasculated flowers, (3) kinds of chemical substances produced by plants such as allelochemicals in plant tissues, soil and air, (4) microorganism flora in soil, (5) overwintering ability, (6) ability to become a weed, (7) the amount of *Agrobacterium* on plants, and (8) kinds of flower-visiting-insects. Cultivation was safely carried out. Since no harmful impact on the environment where the transgenic tomato plants had been cultivated was detected, it is suggested that this tomato strain can be cultivated in an open field. The TMV resistance was maintained throughout generations. These transgenic tomato plants were cultivated in an ordinary field in the summer of 1992 in the campus of the NIAEA.

Discipline: Biotechnology/Environmental science
Additional key words: *Agrobacterium tumefaciens*, gene flow, microbial flora, overwintering ability, plant growth inhibitors

Introduction

A gene producing the coat protein of TMV was introduced into an F_1 plant between *Lycopersicon esculentum*, cultivated type tomato, and *L. peruvianum*, wild type, by F. Motoyoshi and M. Ugaki, the National Institute of Agrobiological Resources (NIAR) in 1988[7]. Trial for evaluating the impact of the release of this tomato plant on the environment were carried out from 1989 to 1992 at the National Institute of Agro-Environmental Sciences (NIAES) in collaboration with the NIAR and the National Agriculture Research Center (NARC). The evaluation was divided into three stages based on the guidelines enacted by the Science and Technology Agency[10] and the Ministry of Agriculture, Forestry and Fisheries[5].

Outline of the experiments is depicted in Fig. 1. The experiments for the first stage were carried out

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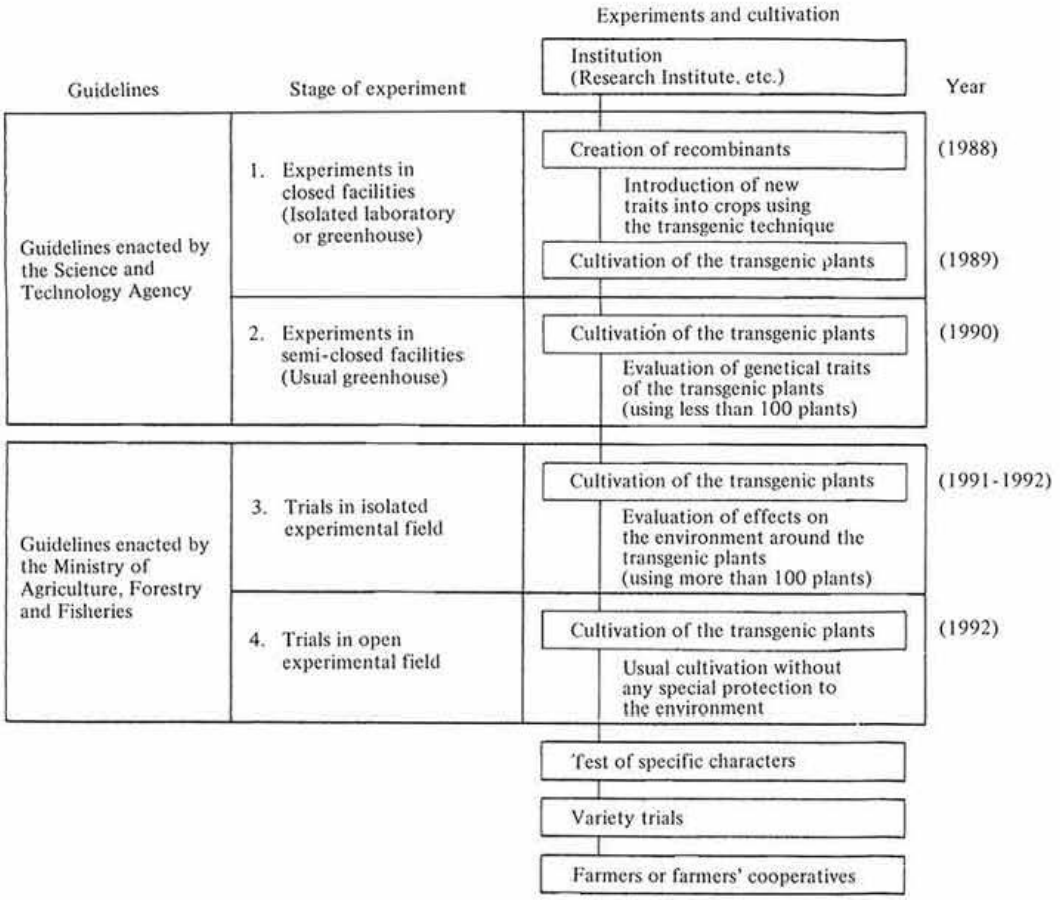

Fig. 1. Flow chart of experiments for evaluating the impact of release of transgenic plants on the environment

in the P2 level greenhouse in 1989, and the experiments for the second stage were initiated in 1990 in a semi-closed greenhouse, where air and small particles such as pollen could enter and exit through screen windows, and terminated in the summer of 1990. The experiments for the third stage were initiated in 1991 and terminated in 1992 in an isolated experimental field surrounded by forests and iron-fences.

The following characteristics were observed in this series of experiments: (1) growth characteristics such as plant height and vigor, (2) pollen dispersion based on fruit set on emasculated flowers, (3) kinds of chemical substances produced by plants such as allelochemicals in plant tissues, soil and air, (4) microorganism flora in soil, (5) overwintering ability, (6) ability to become a weed, (7) the amount of *Agrobacterium* on plants, and (8) kinds of flower-visiting-insects. The evaluation was made by comparing between the transgenic and original plants.

This was the first trial for evaluating the impact of the release of transgenic organisms on the environment in Japan[7]. After these experiments, the transgenic tomato plants were cultivated in an ordinary open field in 1992 at the campus of the NIAES.

(by M. Shiyomi, I. Matsuda and E. Hamaya)

Expression of TMV resistance in the transgenic tomato plants harboring the coat protein gene

Resistance of the transgenic tomato plants to TMV infection was evaluated. TMV was inoculated at

various concentrations to the transgenic and original plants. In the original plants, the amount of propagated viruses in newly developed upper leaves increased rapidly and reached a high level about 5 days after inoculation and decreased thereafter. However in the transgenic plants, the amount of TMV increased very slowly and the total amount was far smaller than that of the original plants at all concentrations (Fig. 2). The number of transgenic plants which showed symptoms was smaller than that of the original plants, and the development of the symptoms was either milder or more delayed in the transgenic plants than in the original plants (Fig. 3). The TMV resistance of the transgenic plants was stronger in the field than in the greenhouse, presumably due to the lower temperature in the field which affected virus propagation. The resistance was stably transmitted to the following generation.

The amount of coat protein in the transgenic plants was 2.5 μg/g fresh weight in the soluble fraction and 20 μg/g fresh weight in the detergent solubilized total protein fraction of the leaves.

(by F. Motoyoshi, M. Ugaki, F. Fukumoto and Y. Ohashi)

Fruit and seed sets in transgenic tomato plants

It was reported that *L. peruvianum* harbors self- and cross-incompatibility genes[11]. Since the

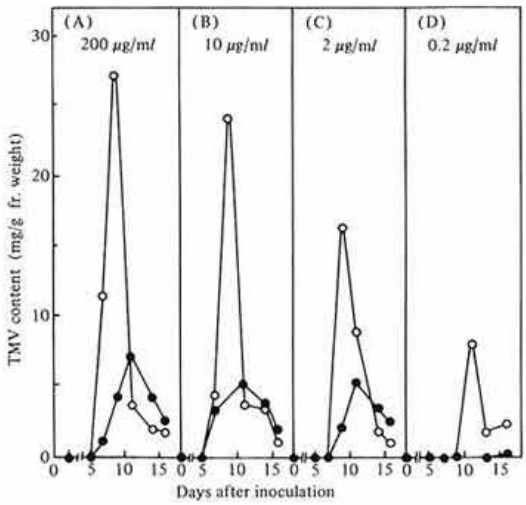

Fig. 2. Inhibition of TMV multiplication on the transgenic tomato plants in the field

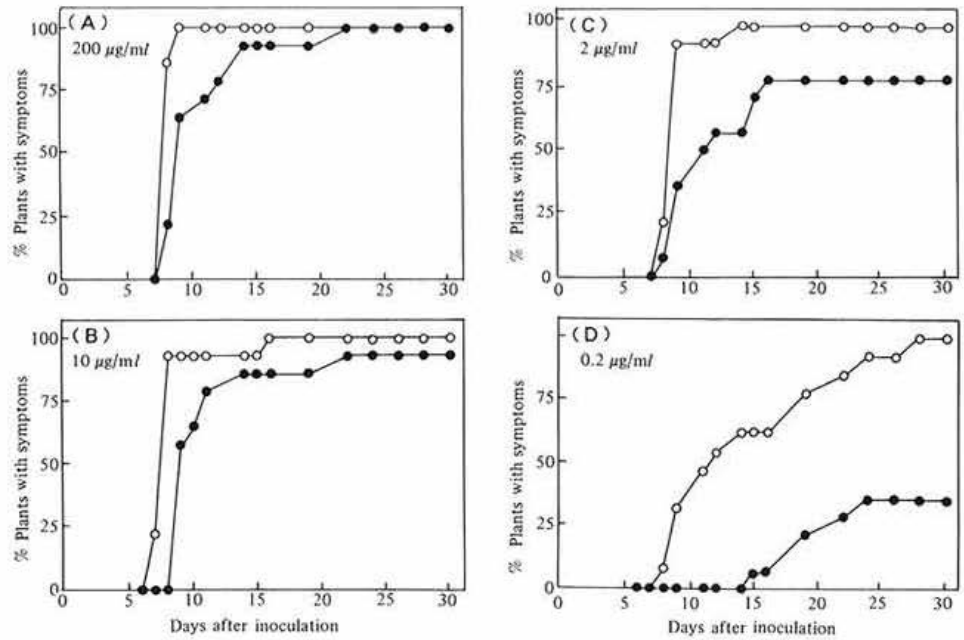

Fig. 3. Resistance to TMV infection of the transgenic tomato plants in the field

Table 1. Number of fruit sets produced by crosses between the transgenic (T), and original plants (O), *L. esculentum* and *L. peruvianum*

Tomato plant		No. of crosses		No. of fruits
Female	Male	Inflorescences	Flowers	
L. esculentum	T	14	14	10
L. esculentum	O	9	9	7
T	*L. esculentum*	22	79	0
O	*L. esculentum*	18	62	0
L. peruvianum	T	11	73	0
L. peruvianum	O	11	72	0
T	*L. peruvianum*	19	63	43
O	*L. peruvianum*	22	72	43
T	O	18	55	0
O	T	19	51	0

transgenic tomato plant was derived from an F_1 plant from a cross between *L. esculentum* × *L. peruvianum*[7], the transgenic plant as well as the original F_1 plant harbored the incompatibility genes. To determine whether any changes in the incompatibility system had occurred during the processes of DNA recombination and subsequent tissue culture, the following eight artificial crossings were made: the transgenic plant × *L. esculentum*, the transgenic plant × *L. peruvianum*, the original F_1 × *L. esculentum* and the original F_1 × *L. peruvianum*, and the reciprocals. Fruit and seed sets were observed in the crosses between *L. esculentum* × the transgenic plant and *L. peruvianum* × transgenic plant, but not in the reciprocals (Table 1). Similar results were obtained for the original F_1.

Also crosses between the transgenic plant × the original F_1 plant and the reciprocals failed to produce fruits. Based on the results obtained it was concluded that the incompatibility system harbored by the *L. peruvianum* plants did not change by mutations.

(by Y. Ukai, Y. Asakawa and M. Shiyomi)

Gene flow from the transgenic tomato plants

To compare the capacity of gene flow through pollen between the transgenic and original plants, anther length, anther size and pollen fertility were examined. No significant differences were observed in these traits between the two types of plants (Table 2). Pollen fertilities were 83.8% and 86.6% for the transgenic and original plants, respectively. No appreciable differences in the number of inflorescences per plant, number of flowers per inflorescence and duration of flowering were recognized between the two types of tomato.

Table 2. Comparison of pollen fertility, anther length and anther size between the transgenic and original tomato plants

	Pollen fertility (%)	Anther length (mm)	Anther size (mm^2)
Transgenic plant	83.8	9.44	22.0
Original plant	86.6	9.30	21.6

Distances of pollen flow from the transgenic and original plants under artificial wind generated by an electric fan in the semi-closed greenhouse were investigated by counting the number of pollen grains trapped on glass slides or fruit sets on emasculated stigmas of cultivated tomato plants (cultivar: Baby). The wind was blown during 30 min every day for 11 days with a velocity of 3 m/sec at maximum at a 1 m distance from the fan. Pollen flow exceeding 120 cm from the source was seldom observed (Table 3) and fruit set by natural crossing was not recorded beyond 76 cm (Table 4). No clear differences in the pollen flow distance were recognized between the transgenic and original plants.

Pollen flow distances were also examined in isolated fields in the third step (Plate 1). Pollen flows beyond 2 m from the source were seldom observed as shown in Table 5. More than 30 flowers for each of the 14 to 15 plants of the cultivated variety, Zuishu, arranged at a distance from 50 to 1,500 cm

from the pollen source were emasculated and subjected to natural crossing with pollen from the transgenic or original plants, and no fruit sets were observed (Table 6).

Longevity of the pollen of the transgenic plants was examined by artificial crossing of emasculated flowers of the cultivated variety with the pollen of the transgenic plants after storage on a glass slide at 25°C in a room. Pollen stored for 48 hr or less produced fruits when used in pollination. The results were in agreement with the findings reported for ordinary cultivated tomato plants.

In conclusion, changes in properties which may be related to the impact on the environment were

Table 3. Distance of pollen flow from the transgenic and original plants in the semi-closed greenhouse

No.	Distance from the pollen source (cm)	No. of pollen grains[a]	
		Transgenic plant	Original plant
0	0	109	29
1	10	39	119
2	32	94	7
3	54	8	32
4	76	14	39
5	98	11	22
6	120	4	9
7	142	7	9
8	164	10	18
9	186	12	4
10	208	4	2
11	230	2	16
12	252	1	6
13	274	7	6

a): Number of pollen grains observed when scanning over a total area of 156 mm^2 on two glass slides.

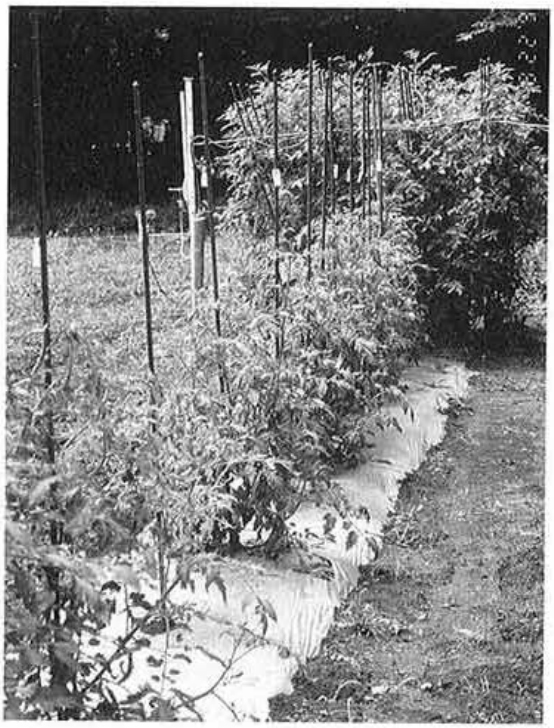

Plate 1. Transgenic tomato plants (right back, taller ones) and cultivated tomato plants (cv. Zuishu: front, shorter ones) grown in the isolated experimental field

Table 4. Number of fruits, on cultivated plants, borne by outcrossing of emasculated flowers by wind-mediated pollen at different distances from the transgenic plants in the semi-closed greenhouse

No.	Distance from the pollen source (cm)	Transgenic plants[a]			Original plants[a]		
		A	B	C	A	B	C
1	10	18	1	3	27	1	4
2	32	28			12		
3	54	18			21	1	1 + (3)[b]
4	76	19			18	1	2
5	98	21			13		
6	120	16			6		
7	142	26	(1)	many[b]	14	(1)	many[b]
⟩	⟩	⟩			⟩		
12	274	26			12		

a): A; Number of flowers emasculated, B; Number of fruits borne, C; Number of seeds per fruits.
b): These cases may be associated with pollen contamination at the time of emasculation.

not recognized in the transgenic tomato plants as compared with the original F_1 plants and ordinary tomato vareities.

(by Y. Ukai, H. Ichikawa, Y. Asakawa and M. Shiyomi)

Production of new plant growth inhibitors in the transgenic tomato plants as well as appearance and growth traits

The production by the transgenic tomato plants of plant growth inhibitors which are not present in the original nontransgenic tomato plants and may exert a harmful influence on the environment was examined. The following characteristics were compared between the transgenic and original tomato plants: (1) contents of phenolic acids, generally considered as allelochemical substances, in the plant body, (2) presence of plant growth inhibitors secreted from the roots into the soil, (3) presence of volatile compounds released from the plants to the atmosphere, and (4) germination and growth of cucumber plants in the soil used for the cultivation of the transgenic or original tomato plants and in the soil mixed with a dry powder of these tomato plants as shown in Fig. 4.

The results indicated that there were no differences between the transgenic and original tomato plants.

Table 5. Distance of pollen flow from the transgenic and original tomato plants in the isolated field

Glass number[b]	Distance from the pollen source (m)	Number of pollen grains[a]			
		Transgenic plants		Original plants	
		N[b]	S[b]	N	S
1	0.25	7	11	2	1
2	0.75	0	0	1	0
3	1.25	0	0	1	1
4	1.75	0	1	0	11
5	2.25	0	0	0	1
6	2.75	0	0	0	0
7	3.25	-[c]	0	-	0
8	3.75	-	-	-	0
Total		7	12	4	14

a): The number of pollen grains was indicated by the count observed in a specific area (18 × 8 mm) on a glass side.

b): Glass slides were arranged on each of the northern (N) and southern (S) sides of 14 transgenic and original tomato plants, and were numbered in order of distance from the plants.

c): Not counted.

Table 6. Number of fruit sets produced by outcrossing between a tomato cultivar Zuishu and emasculated flowers of the transgenic (T) or original (O) plants

Field number	Area (are)	Pollen donor	Total number of flowers of pollen donor		Number of emasculated flowers	Number of fruits by outcrossing
			Start[a]	End[a]		
11	7	T	250	450	252	0
11	7	O	150	320	208	0
10	3	T	140	210	300	0
12	3	O	150	200	257	0
Total					1,017	0

a): Approximate number of total flowers in each of 14 pollen donor plants (T and O) at the start and end of emasculation of cultivated tomatoes.

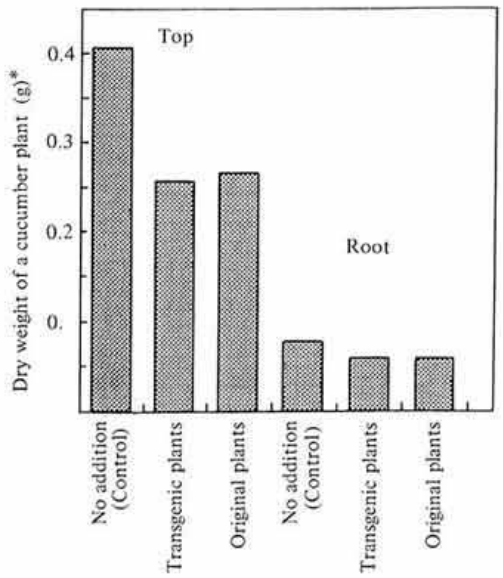

Fig. 4. Growth in closed greenhouse of cucumber plants in soil mixed with a dry powder of the transgenic or original tomato plants

Namely, no additive compounds related to plant growth inhibition were detected in the transgenic tomato plants into which foreign genes had been introduced.

No differences were observed in the plant appearance and various growth characteristics between the transgenic plants and the original tomato plants grown in the closed greenhouse, the semi-closed greenhouse and the isolated experimental fields (Plate 2).

(by Y. Asakawa)

Survival of *Agrobacterium tumefaciens* on the transgenic tomato plants

Agrobacterium tumefaciens was used as a plant vector for the construction of the transgenic tomato plants. Survival of this bacterium on plants was examined in specimens from the closed greenhouse and semi-closed greenhouse. Microorganisms isolated by shaking or homogenized specimens in sterile distilled water were plated on several media including a selective medium YEB (beef extract 5 g, yeast extract 1 g, bactopeptone 1 g, sucrose 5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 20 g, distilled water 1,000 ml) supplemented with streptomycin (200 ppm) and rifampicin (5 ppm).

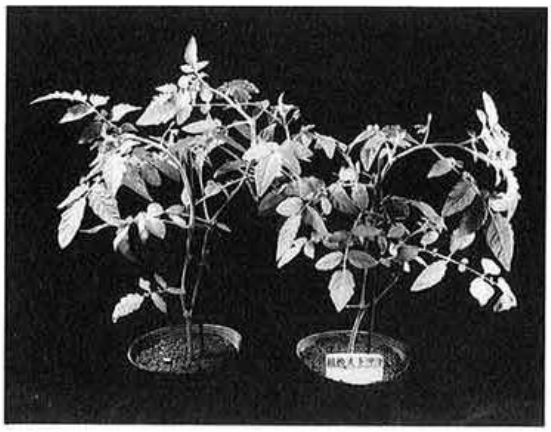

Plate 2. Appearance of the original (left) and transgenic (right) tomato plants grown in the closed greenhouse

Strain PC2760 of *Agrobacterium tumefaciens* was not detected in any plant samples (leaves and stems) collected from the greenhouses used for the first and second stages of the experiments. It was thus confirmed that *Agrobacterium tumefaciens* had not survived on the plants.

(by M. Sato)

Changes in microbial flora in soil used for the cultivation of the transgenic tomato plants

The effect of the release of transgenic tomato plants on the soil microflora was investigated in the isolated experimental field in the third stage of the experiment. The numbers of microbes, bacteria, actinomycetes and fungi in soil were monitored periodically by the viable count method from July 25, 1990, to August 28, 1991 (Fig. 5). The viable count method had been used for monitoring the genetically modified microorganisms released in soil in both the U.S. and the Netherlands[4,13] and was considered to be sufficiently reliable.

The microflora was compared between the soils in which the transgenic tomato and original tomato plants had been planted. The amount of microbes in the soil cultivated with the transgenic tomato plants was slightly larger than that in the soil cultivated with the original tomato plants just after transplanting. However, the differences were not statistically significant. It was, therefore, concluded that there were no significant differences in the

plate counts of bacteria, actinomycetes and fungi between the soils in which the transgenic and original tomato plants were grown.

(by A. Hasebe and K. Yokoyama)

Insects visiting the transgenic tomato flowers

Visits to transgenic tomato flowers by only four wild species of halictine bees, *Lasioglossum occidens*

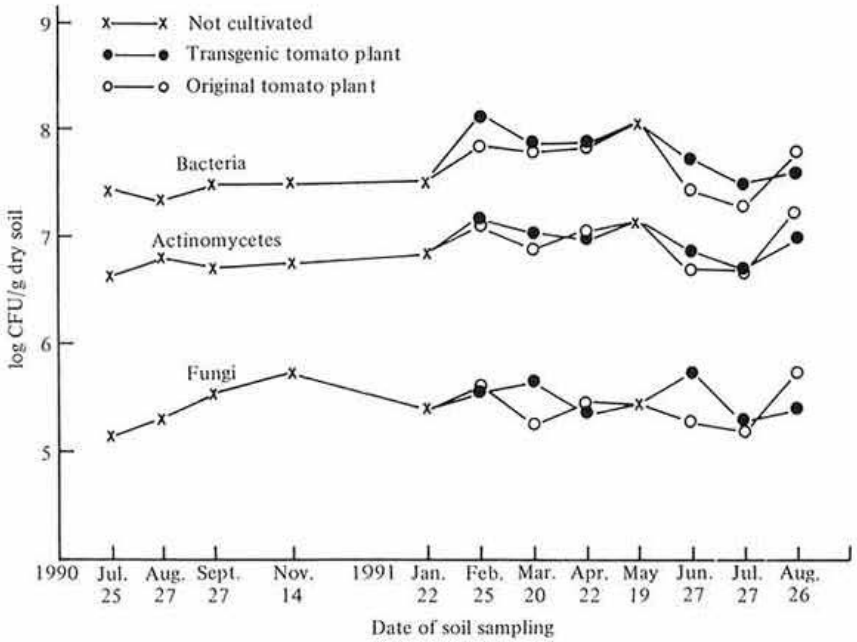

Fig. 5. Microflora changes in the soil where the transgenic and original tomato plants were cultivated

Table 7. Growth of tomato plants after removal of transparent polyethylene film

Tomato plant	Color[b]	February 25 (+10)[a]			March 19 (+32)[a]		
		Top	Underground		Top	Underground	
			Stem	Root[c]		Stem	Root
Transgenic	Green	0[d]	5	0	0	1	0
	White	0	0	5	0	0	1
	Yellow	0	0	0	0	0	0
	Yellow to brown	5	0	0	0	3	3
	Brown	0	0	0	5	1	1
Original	Green	0	5	0	0	1	0
	White	0	0	5	0	0	1
	Yellow	0	0	0	0	0	0
	Yellow to brown	5	0	0	0	4	4
	Brown	0	0	0	5	0	0

a): Days after removal of film, b): Result of visual observation,
c): Color of inside of underground parts, d): Number of plants, with a given color, out of five plants.

Table 8. Summary of experiments for evaluating the impact of the release of transgenic tomato plants with TMV resistance on the environment

Item	1st stage (closed greenhouse)	2nd stage (semi-closed greenhouse)	3rd stage (isolated experimental field)	References	Remarks
① Origin and taxonomy of the materials				× [3,8]	
② Traits relating to reproduction					
Natural crossing rate				× [9,11]	0.5 to 4% in literature
Fertilization by wind		×	×		Minimal
Fertilization by insects		×	×		Nil or minimal
Longevity of pollen			×		Within 2 days
Distance of pollen flow		×	×		About 10 m (by electric fan)
Compatibility and fruit-bearing	×	×	×		Incompatible in both the original and transgenic tomato plants
Fertility of pollen	×		×		84 to 87% for both the original and transgenic tomato plants
Insect pollinators			×		Four wild species of halictine bees
③ Traits relating to growth	×	×	×		No differences between the original and transgenic tomato plants
④ Cold resistance and over-wintering			×	× [3]	No ability to overwinter
⑤ Examination of production of plant growth chemical inhibitors					
Compounds in plant bodies	×	×			No differences between the original and transgenic tomato plants
Volatile substances from leaves	×	×			
Exudation from roots	×				
Plant growth in mixture of plant bodies in the soil	×	×			
⑥ Survival of *Agrobacterium tumefaciens*					
Amount in soil	×	×			No differences between the original and transgenic tomato plants
Amount on upper leaves and stems	×	×			
Amount on lower leaves and stems	×	×			
⑦ Microbial flora in soil		×	×		No differences between the original and transgenic tomato plants
⑧ Vegetation around the isolated experimental field			×	× [12]	No plant species with ability to cross with tomato plants
⑨ Resistance of the transgenic tomato plants to TMV	×		×		Resistant to TMV
⑩ Detection of coat protein	×		×		Detected from the transgenic tomato plants

× indicates items examined.

and other three species of the same genus, were observed in the isolated experimental field, suggesting that these small bees were able to pollinate tomato flowers among plants within a short distance. There were no differences in the visits between the transgenic and original tomato plants. Although European and Japanese honey bee colonies were reared at a distance of 300 m from the area, no honey bees visited the tomato flowers.

Campbell[1] showed that distances over which insect pollinators could disseminate pollen were generally very short.

(by T. Matsumura)

Vegetation around the isolated experimental field used for the cultivation of the transgenic plants

Vegetation in the isolated experimental field used for the cultivation of the transgenic tomato plants was investigated. Fifty woody species belonging to 28 families and 92 herbaceous species belonging to 37 families were listed[7]. No plant species which could be crossed with tomato plants were observed.

(by K. Noguchi)

Overwintering ability of the transgenic tomato plants

The overwintering ability of the transgenic and original tomato plants in the open air was investigated in the isolated experimental field. Twenty transgenic tomato plants were transplanted in the isolated experimental field covered with a transparent polyethylene film and mulched with a black polyethylene film on February 6, 1991. After rooting, the film covering the tunnel was removed and the tomato plants were left in the open air. The results are shown in Table 7. The color of the top parts of the tomato plants turned yellow or brown due to cold air during 10 days after film removal, while that of the underground parts remained green or white and these parts were considered to be still alive. Although the change in the color of the underground parts occurred a few days later as compared with the leaves and stems, whole transgenic tomato plants died sometime about a month after the removal of the polyethylene film.

There were no differences in the symptoms between the transgenic tomato plants and original ones. It was reported that the optimum temperature for growing tomato plants is 20–25°C, the minimum temperature, 5–10°C, and frost injury occurs below 1°C[6]. Usually the minimum air temperature in winter is below zero in most areas in Japan. These results suggest that the transgenic tomato plants could not overwinter, namely that their growth characteristics did not change from annual to perennial.

(by K. Noguchi)

Conclusion

The experimental results are summarized in Table 8. From this table, the following conclusions were drawn: (1) experiments and cultivation, as stated in the previous sections, were safely carried out throughout the three stages; (2) since no harmful impact on the environment where the transgenic tomato plants had been cultivated was detected, it is suggested that this tomato strain can be cultivated in ordinary open fields; and (3) the TMV resistance was maintained throughout generations.

For further development of a safety evaluation test, the following problems remain to be solved. First, the behavior of genetic materials, such as pollen and seeds, of transgenic plants in the open field should be a matter of concern. Eco-genetical investigations regarding the dispersion and changes in the frequency of the transferred gene in the population should be carried out. Second, it is important to develop a new technology to replace the use of genes producing antibiotics such as kanamycin, as marker genes in order to obtain safe transgenic plants, especially for food use.

(by M. Okada and M. Shiyomi)

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(Received for publication, Nov. 30, 1992)