Resistance Mechanism of Cultured Plant Cells to Tobacco Mosaic Virus (II) Resistance of Calli, Protoplasts, Cotyledons, Leaf Discs and Intact Leaves of Tomato Plants*

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豊田秀吉*・山本百与*・平井篤造*: タバコモザイクウイルスに対する植物培養細胞の抵抗性発現機作 (第2報) トマトのカルス細胞, プロトプラスト, 子葉, リーフディスクおよび本葉における TMV 抵抗性発現の比較

Abstract

Resistance of tomato against TMV was examined by using various plant materials, such as callus tissues, protoplasts from callus tissues or leaves, leaf discs, cotyledons and intact leaves of resistant tomato plants carrying *Tm-2²* gene. TMV multiplication in these materials was estimated by means of quantitative immunoelectrophoresis using FITC-conjugated antibody. An amount of TMV, after incubation of inoculated callus tissues on solid medium for 6 days, was much higher in callus tissues of susceptible tomato plant than of resistant plants. The difference in these two types of callus tissues was more distinct in the largest pieces of callus tissues than in smaller ones, which were produced when they were inoculated with TMV by shaking. In the smallest pieces of callus tissues, there was no difference between callus tissues prepared from susceptible and resistant plants. In protoplasts, there was no significant difference between resistant and susceptible plants. On the other hand, a significant difference was detected in leaf discs, cotyledons and leaves of both plants. These results indicated that resistance gene, *Tm-2²*, of tomato against TMV functioned effectively in large pieces of callus tissues as well as leaf discs and other intact materials of tomato, but not in protoplasts.

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Key Words: resistance expression, tomato callus tissues, TMV.

Introduction

Resistance genes of tomato against tobacco mosaic virus (TMV) have been identified and the mode of inheritance has also been widely investigated in various tomato plants^{1,13)}. However, the decisive reactions controlled by these genes have not been clarified. Using a system of TMV and protoplasts prepared from tomato leaves carrying resistance genes, Motoyoshi and Oshima^{5,6)} investigated the expression of these resistance genes and noted that the mode or timing of resistance expression was different among genes, *Tm-1* and *Tm-2* or *Tm-2²*. They have also discussed that the expression of resistance controlled by *Tm-2* or *Tm-2²* was associated with host cellular connection and interaction⁶⁾. In addition to virus-protoplast system, Murakishi and his

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colleagues indicated that callus tissues of tomato⁷⁾ and tobacco^{8,9,12)} were useful system for investigating TMV multiplication, because of high efficiency of infection and high titer of the virus. They have also showed the possible application of tobacco callus tissues to the investigation of resistance reaction such as hypersensitivity found in intact tobacco plants inoculated with TMV^{2,3)}. The use of callus tissues may be interest system for studying the resistance mechanisms in which host cellular connection and interaction were required.

To investigate the resistance expression of gene, *Tm-2²* (*Tm-2^a*), of tomato against TMV at a cellular level, it was designed in the present study to inoculate callus tissues, protoplasts, cotyledons, leaf discs and leaves obtained from resistant and susceptible tomato plants with TMV and to compare the resistance expression among them.

Materials and Methods

Plant and virus. GCR 267, which was gratefully provided by the late Dr. K. Noba, Vegetable and Ornamental Crops Research Station, was a breeding line carrying homozygous *Tm-2²* which originated from a wild species, *Lycopersicon peruvianum*¹⁾. Zuiko (ZK) was a cultivar of *L. esculentum* which was suggested by Yamakawa¹⁶⁾ to carry *Tm-2²* heterozygously. Both lines were resistant to TMV. Fukuju No. 2 (F 2), a cultivar of *L. esculentum*, was used as a susceptible plant to TMV. The seeds of ZK and F 2 were purchased from Sakata Shubyo Co., Ltd, Yokohama, Japan.

TMV (strain OM) was purified from tobacco leaves (*Nicotiana tabacum* cv. Bright Yellow) by four cycles of differential centrifugation (10,000×*g*, for 20 min and 100,000×*g*, for 90 min), lyophilized and stored at -20 C.

Callus culture. Callus was induced by the method reported previously¹⁴⁾. Seeds of tomato sterilized with 10 % sodium hypochlorite for 10 sec and EtOH for 10 min were germinated and then incubated on Murashige-Skoog (MS) medium¹⁰⁾ solidified with 0.8 % agar, adjusted to pH 5.8 and supplemented with 0.5 µg/ml sodium salt of 2,4-dichlorophenoxyacetic acid (2,4-D·Na) and 0.05 µg/ml kinetin at 26 C under continuous illumination of 3,000-4,000 lux. Induced callus tissues were transferred to liquid MS medium (2,4-D·Na at 0.1 µg/ml and kinetin at 0.05 µg/ml) and shaken at 110 rotations/min to obtain friable calli. The calli were subcultured for several passages on the agar medium and used for the following experiments.

Protoplast preparation. *Callus tissues:* Callus tissues were cultured in the liquid MS medium for 5 days and incubated at 30 C for 2 hr in 5 ml of enzyme solution containing 40 mg/ml Cellulase Onozuka RS (Yakult Pharmaceutical Industry Co., Ltd, Nishinomiya, Japan), 10 mg/ml Pectolyase Y-23 (Seishin Pharmaceutical, Co., Ltd, Tokyo, Japan) and 0.4 M mannitol (pH 7.0). After incubation, the solution was passed through stainless sieves with 200 and successively 50 µm diameter of pore. Filtrates were centrifuged at 100×*g* for 5 min and the pellets were resuspended in 0.5 M mannitol. Isolated protoplasts were obtained after washing the pellets with the mannitol solution by repeated centrifugation at 100×*g* for 5 min.

Leaves: Tomato protoplasts were prepared according to the two step procedure reported by Motoyoshi and Oshima⁵⁾. Using 20 tomato leaves grown at 26 C for 30-40 days, the lower epidermis of their leaflets was peeled off with forceps and digested

at the first step with 50 ml of enzyme solution containing 5 mg/ml Macerozyme R-10 (Yakult Pharmaceutical Industry Co., Ltd, Nishinomiya, Japan), 1 mg/ml potassium dextran sulphate and 0.7 M mannitol (pH 7.0) and subsequently with 50 ml of the solution containing 10 mg/ml Cellulase Onozuka R-10 and 0.7 M mannitol. After centrifugation, the pellet of protoplasts was resuspended in 0.7 M mannitol and washed twice with the same solution.

Inoculation of TMV. *Callus tissues*: After shaking incubation with liquid MS medium for 5 days, the tissues were filtered through a 2,000 μ m sieve, weighed (1,000 mg) and shaken at 350 strokes/min for 15 min with 3 ml of MS medium containing 250 μ g TMV. After TMV inoculation in shaking medium, the callus tissues were washed twice with fresh MS medium. A part of the tissues was homogenized soon after inoculation to assay the amount of TMV adhered to them, and the rest was incubated on solid MS medium for 6 days.

Protoplasts: Protoplasts isolated from both calli and leaves were inoculated with TMV and incubated by the method of Motoyoshi and Oshima⁵⁾ except that mannitol concentration was adjusted to 0.5 M for callus protoplasts. The final concentrations were 1 μ g/ml for TMV, 1 μ g/ml for poly-L-ornithine and 2 to 4×10^5 /ml for protoplasts in 0.01 M phosphate buffer (pH 6.7) containing mannitol. Rate of infected protoplasts was measured 48 hr after inoculation by the fluorescent antibody staining technique developed by Otsuki and Takebe¹¹⁾.

Cotyledons, leaves and leaf discs: Cotyledons and leaves of tomato plants were inoculated with TMV at 15 and 20 days after sowing, respectively. Upper surface of them was gently rubbed with inoculum solution (1 mg TMV/ml) containing 0.2 mg/ml carborundum (600 mesh), washed with water and incubated at 26 C for 6 days under continuous illumination of 6,000–8,000 lux. Discs (4 mm in diameter) were punched out from the inoculated leaflets with a cork borer 30 min after inoculation and floated on water for 6 days under the same conditions mentioned above.

TMV estimation. TMV amounts in the inoculated plant materials were estimated by means of quantitative immunoelectrophoresis using FITC-conjugated antibody reported previously¹⁵⁾. Inoculated materials were weighed and homogenized with 5 parts of water (V/W) and then crude homogenates were clarified by centrifugation ($1,500 \times g$, for 20 min). Ten μ l of homogenates were charged to the wells of 1 % agarose gel containing 0.1 % (v/v) FITC-conjugated antibody (from 1 % stock solution, w/v) and electrophoresed in Tris-HCl buffer (pH 8.6) at 4 C, for 5 hr under a constant voltage of 8 V/cm. TMV amounts were calculated from the migration distance of antigen-antibody complex detected under UV-irradiation.

Results

TMV amounts in callus tissues

TMV amounts were estimated soon after inoculation of the tissues derived from the resistant and susceptible tomato plants. The amounts of TMV adhered to the tissues of resistant tomato plants, GCR 267 and ZK, were 0.003 and 0.005 mg/g fresh weight of callus, respectively and 0.006 mg/g fresh weight of callus of susceptible plant (F2). It indicated that there was no significant difference in the amounts of

Fig. 1 TMV amounts in callus tissues prepared from resistant tomato plants, GCR 267 and Zuiko (ZK), and a susceptible one, Fukuju No. 2 (F2), 6 days after inoculation by shaking method.

Fig. 2 Dissociation of F2 callus tissues after 15 min-shaking. Numbers in the figure reveal diameters (μm) of callus tissues.

Fig. 3 TMV amounts in each size class of callus tissues 6 days after inoculation with TMV.

Three classes of callus tissues were obtained by 15-min shaking and filtration through sieves, indicating that A, B and C in the figure reveal callus tissues with larger than 1,000 μm , 500 to 1,000 μm and less than 500 μm in diameter, respectively.

adhered TMV among three callus tissues. After 6 days-incubation, however, TMV amount of F2 callus was 5–9 times larger than that of resistant calli, but any difference was not found between calli of GCR 267 and ZK (Fig. 1).

By shaking for 15 min, the inoculated callus tissues were dissociated into smaller clumps of various sizes, as shown in Fig. 2. Three size classes of clumps were separately incubated for 6 days and then TMV amounts of them were measured (Fig. 3). The highest amount of TMV was found in the largest clumps (larger than 1,000 μm in diameter) of F2 callus tissues. In the middle size of clumps (500–1,000 μm in diameter), considerably large amount of TMV was also detected, but it was not so high as the largest one of F2. Any significant difference in TMV amounts was not found among the smallest callus tissues of three different lines. In comparison of TMV amounts between GCR 267 and ZK, there was no

significant difference in relation to callus sizes. Callus fresh weight during 6 days-incubation period increased 1.34, 1.28 and 1.33 times in the largest, middle and the smallest size pieces of F2 callus, respectively. The similar tendency was observed in resistant ones.

TMV amounts in protoplasts

Infection rates and amounts of TMV were examined in protoplasts prepared from callus tissues and leaves of resistant (ZK) and susceptible (F2) plants (Table 1). Leaf protoplasts from both resistant and susceptible lines were infected and similar amounts of TMV were produced in both types of leaf protoplasts. In protoplasts prepared from calli of GCR 267, ZK and F2, no significant difference in infection rate and the amount of TMV was found, although both values were much lower in protoplasts of callus than that of leaf.

TMV amounts in cotyledons, leaf discs and leaves

The amounts of TMV were similar among leaf discs, cotyledons and intact leaves

Table 1 TMV amounts^{a)} in protoplasts prepared from callus tissues and leaves of tomato plants

Tomato plants	Origin of protoplasts	No. of protoplasts/ml	% infection	TMV amounts (mg/g fresh weight of protoplast)
GCR 267	Callus	3.2×10^5	18.8	0.12
	Leaf	—	—	—
Zuiko	Callus	2.6×10^5	12.0	0.09
	Leaf	3.4×10^5	54.3	0.74
Fukuju No. 2	Callus	3.9×10^5	14.4	0.10
	Leaf	3.3×10^5	42.3	0.71

a) TMV amounts and % infection were examined 48 hr after inoculation.

Fig. 4 TMV amounts in leaf discs (LD), cotyledons (CD) and leaves (L) of three tomato cvs. 6 days after inoculation.

taken from susceptible line. These amounts were higher than those from resistant (GCR 267 and ZK) lines (Fig. 4). On the other hand, no significant difference was found between homozygous GCR 267 for *Tm-2²* and heterozygous ZK for the gene, when callus- and leaf-protoplasts, leaf discs, cotyledons and intact leaves were examined.

Discussion

In the present experiment, the resistance expression of tomato carrying a resistant gene (*Tm-2²*) against TMV was examined in various levels of plant materials. The result obtained is summarized in Table 2, in which the resistance is shown on the basis of TMV concentrations in both resistant and susceptible plant materials. The resistance by *Tm-2²* was expressed in leaf discs, cotyledons and leaves of resistant tomato plants, GCR 267 and ZK, and the grade of resistance in both plants was at the similar level. These results suggest that *Tm-2²* effectively functioned at a heterozygous state. A complete dominance of *Tm-2²* has been demonstrated to Ohio strains of TMV by Cirulli and Alexander⁴⁾ and Alexander¹⁾.

To elucidate the resistance mechanism of tomato against TMV infection, callus tissues and protoplasts were inoculated with TMV. Inoculation of callus tissues with TMV has showed that the tissues expressed resistance at the same rate as the intact plants⁷⁾ and that tobacco callus tissues carrying *N*-gene formed necrotic lesions after inoculation²⁾. The present experiment showed that increase in TMV concentration during the incubation was about 120-fold in susceptible callus tissues and 30- to 40-fold in resistant ones. Therefore, the resistance was expressed in callus tissues from tomato plants with *Tm-2²*. In the present method, the callus tissues were inoculated with TMV by strong shaking. It brought about the dissociated callus clumps and enable us to estimate separately TMV concentration in the classified clumps. A difference in TMV concentration was detected between the largest pieces of callus tissues of resistant and susceptible plants. In contrast, TMV concentration in the

Table 2 Comparison of resistance expression in various materials from tomato plants against TMV

Materials	Evaluation ^{a)} for TMV multiplication		
	GCR 267 (<i>Tm-2²/Tm-2²</i>)	ZK (<i>Tm-2²/+</i>)	F 2 (<i>+/+</i>)
Leaf protoplasts	not examined	+++	+++
Callus protoplasts	(+) ^{b)}	(+)	(+)
Callus tissues smaller size	+	+	+
larger size	+	+	+++
Leaf discs	+	+	+++
Cotyledons	+	+	+++
Leaves	+	+	+++

a) Based on TMV amounts, which were classified into two types; one was at a higher level of TMV amounts (more than 0.4 mg/g fresh weight) and the other at a lower level (0.1-0.2 mg/g fresh weight).

b) TMV amounts were much lower than those with leaf protoplasts, but very similar among three plants.

smallest pieces of callus tissues (0.1–0.2 mg/g fresh weight of callus) derived from resistant plants was similar to those in the largest pieces and also to those in other materials prepared from the corresponding intact plants. Thus, *Tm-2*² effectively functioned and expressed the resistance in the callus tissues without relation to their sizes. In the smallest pieces of F2 callus, however, the levels of TMV concentrations were as low as those in resistant ones. Using a system of tomato leaf-protoplasts and TMV, Motoyoshi and Oshima⁶⁾ showed that *Tm-1* effectively functioned to suppress the multiplication of TMV in contrast to *Tm-2* and *Tm-2*². Similar results were obtained in the present study with respect to *Tm-2*² carrying protoplasts prepared from callus tissues as well as tomato leaves. These results support the results of Motoyoshi and Oshima⁶⁾ that *Tm-2* and *Tm-2*² expressed resistance which requires cell- to cell-connection and interaction as a phenotypic effect. Thus, *Tm-2*² was unable to suppress completely the multiplication of TMV and therefore allowed TMV to multiply to a limited level in plant materials as used in the present study. The authors, therefore, suppose that *Tm-2*² did not effectively function to suppress TMV-multiplication in cells infected primarily and that it may either suppress the translocation of TMV from primarily infected to adjacent cells, or induce the secondary induction of some reaction against TMV-multiplication.

In callus tissues and protoplasts, definite conditions for TMV inoculation and multiplication are important for comparing TMV concentrations in resistant and susceptible plants. The amount of TMV multiplied in F2 callus tissues was a half of intact leaves of the corresponding plants, and low than that in inoculated tobacco callus tissues⁹⁾. The lower titer of TMV obtained in the tomato callus may partly be attributed to the differences in chemical compositions of culture media used before or after inoculation, as discussed by Zaitlin and Beachy¹⁷⁾. Infection efficiency and TMV concentrations from leaf protoplasts were significantly high⁵⁾, but those of protoplasts from callus tissues were much lower than those from leaves. Thus, the conditions for inoculating callus protoplast or the incubation should be further examined.

Literature cited

1. Alexander L. J. (1971). *Phytopathology* 61: 611–617.
2. Beachy, R. N. and Murakishi, H. H. (1971). *Ibid.* 61: 877–878.
3. Beachy, R. N. and Murakishi, H. H. (1973). *Virology* 55: 320–328.
4. Cirulli, M. and Alexander, L. J. (1969). *Phytopathology* 59: 1287–1297.
5. Motoyoshi, F. and Oshima, N. (1975). *J. gen. Virol.* 29: 81–91.
6. Motoyoshi, F. and Oshima, N. (1975). *Ibid.* 34: 499–506.
7. Murakishi, H. H. (1968). *Phytopathology* 58: 993–996.
8. Murakishi, H. H., Hartmann, J. X., Pelcher, L. E. and Beachy, R. N. (1970). *Virology* 41: 365–367.
9. Murakishi, H. H., Hartmann, J. X., Beachy, R. N. and Pelcher, L. E. (1971). *Ibid.* 43: 62–68.
10. Murashige, T. and Skoog, F. (1962). *Physiol. Plant.* 15: 473–497.
11. Otsuki, Y. and Takebe, I. (1969). *Virology* 38: 497–499.
12. Pelcher, L. E., Murakishi, H. H. and Hartmann, J. X. (1972). *Ibid.* 47: 787–796.
13. Pelham, J. (1966). *Euphytica* 15: 258–267.
14. Toyoda, H., Tanpo, H., Kunita, T. and Hirai, T. (1983). *Mem. Fac. Agr. Kinki Univ.* 16: 31–40.
15. Toyoda, H., Ooishi, Y. and Hirai, T. (1983). *Ann. Phytopath. Soc. Japan* 49: 488–494.

16. Yamakawa, K. (1978). Nogyo oyobi Engei 53: 1151-1154.
17. Zaitlin, M. and Beachy, R. N. (1974). Adv. Virus Res. 19: 1-35.

和文摘要

タバコモザイクウイルスに対する植物培養細胞の抵抗性発現機作 (第2報) トマトのカルス細胞, プロトプラスト, 子葉, リーフディスク および本葉における TMV 抵抗性発現の比較

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タバコモザイクウイルス (TMV) 抵抗性遺伝子 *Tm-2^s* をもつトマト GCR 267, 瑞光 (ZK) と罹病性の福寿2号 (F2) から, カルス細胞, プロトプラスト, 子葉および本葉をとり, それぞれに TMV (OM系) を接種し抵抗性の発現状況を検討した。TMV の定量には免疫電気泳動法を使用した。TMV を強振接種法でカルスに接種し, 6 日後に TMV を定量したところ, F2 のカルスでは顕著な TMV 量の増加が認められたが, 抵抗性トマトから得たカルスでは F2 の 1/5 から 1/9 に抑えられていた。また強振接種法により分離したカルスをその大きさに類別し TMV 量を調べたところ, 直径が 500 μm 以上の F2 のカルスでは TMV 量の有意な増高が認められ, それ以下のカルスでは罹病性と抵抗性のカルスの間に差は認められなかった。抵抗性および罹病性トマトのカルスまたは本葉からプロトプラストを調製し, TMV を接種したところ, いずれのプロトプラストでも, 48時間後の TMV 感染率や TMV 量には抵抗性と罹病性の間で差は認められなかった。子葉, 本葉および本葉のリーフディスクの TMV の量を比較した場合, 抵抗性トマトでは TMV の増殖が抑えられていたが, GCR 267 と ZK の間では TMV 量に有意な差は認められなかった。