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Leakage of Cell Constituents Associated with Local Lesion Formation on *Nicotiana glutinosa* Leaf Infected with Tobacco Mosaic virus

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大橋祐子*・下村　徹*：タバコモザイクウィルスに感染した
Nicotiana glutinosa 葉での局部病斑の形成に伴なう
細胞内液の漏出について

Abstract

The leakage of cell constituents from tobacco mosaic virus-infected *N. glutinosa* leaf was determined by the increase in conductivity of the leaf wash or by the loss of ^{32}P from the tissues which had been fed with ^{32}P phosphate.

The leakage occurred with the appearance of visible symptoms and the rate increased with the enlargement of the lesions. Lesions induced by transfering leaves from 30C to 22C, by heat or cold shock treatments of *N. glutinosa* leaves systemically infected at 30C also caused the leakage just prior to or during the appearance of visible symptoms and its rate usually decreased with the completion of necrotic lesions.

These results suggest that the leakage of cell constituents is an early host response to lesion formation in hypersensitive hosts. (Received December 4, 1975)

Fig. 1. Tiny sunken colourless spots appeared on the lower surface of *N. glutinosa* leaf, the upper surface of which had been inoculated with TMV 2 days before the appearance (22C).

Introduction

When the upper surface of the leaves of Samsun NN tobacco or *Nicotiana glutinosa* is inoculated with tobacco mosaic virus (TMV), local lesions are first detectable as tiny sunken colourless spots, which are only visible on the lower surface of the inoculated leaves (Fig. 1). Under high humidity, sometimes small water droplets are formed around the developing lesions on the upper surface. These observations suggest that alterations of the integrity of cell membranes, leakage of cell constituents, and subsequent cellular collapse are early host responses in lesion formation of local lesion hosts.

When detached leaves of *N. glutinosa* inoculated with TMV are incubated at high temperature (30C), TMV spreads "systemically" and no lesions appear even on the inoculated leaves. However, when

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such leaves are transferred to a low temperature (22C), the infected tissues collapse and form necrotic lesions[11,26]. Similar necrotic lesions were induced by heat or cold shock treatment[19,20,27], actinomycin D or chromomycin A_3 treatment[21], UV irradiation[22], and dark treatment[23] of TMV-inoculated *N. glutinosa* leaves which had been incubated at 30C. By these treatments, water-soaked areas appeared as the initial infection sites, subsequently these areas collapsed and resulted in the necrotic lesion formation during incubation at 30C, although most of the induced lesions were necrotic rings with green centers instead of solid necrotic lesions on the non-treated leaves incubated at 22C. Sometimes, small water droplets were seen on the surface of the lesions.

In bacterial[5, 8] or fungal[9, 14] diseases, an increase in cell permeability in infected tissues during the infection process has been reported. This study was undertaken to determine whether the leakage of cell constituents occurs during lesion formation on the leaves of *N. glutinosa* inoculated with TMV, including necrotic lesions induced by the various temperature treatments.

Materials and Methods

Plants and virus. *Nicotiana glutinosa* plants were grown in a greenhouse (20—30C) for 2.5—3.0 months after seeding. Fully expanded leaves were inoculated 2 days after removal of the terminal bud with purified preparations (10 μg/ml) of the common strain of tobacco mosaic virus(TMV).

Measurement of electrolyte leakage. Detached leaves were cut into halves, one of the halves was inoculated with TMV and another halves served as a non-inoculated control. The leaves were placed in petri dishes and incubated at 22C under continuous illumination of 6,000 Lux from fluorescent lamps. At appropriate time intervals, each half leaf was washed with 5 ml of deionized water using a "Komagome" transfer pipette. The leaf wash was collected and subjected to conductivity measurement by a conductivity bridge (Model CD-32M, M&S Instruments Inc.) using a cell (CDS-12, cell constant value ; 0.1).

In lesion induction studies, TMV-inoculated plants were incubated at 30C for 2 days under continuous light, then the inoculated leaves were detached and cut into halves. One of the halves was subjected to the various temperature treatments and another half was incubated at constant 30C as a control. Non-inoculated half leaf was incubated at the same temperatures and served as another control. After the treatments, the leaves were placed in petri dishes and incubated at 30C. At appropriate time intervals, each half leaf was washed with deionized water and the conductivity of leaf wash was determined as described above. The readings were corrected by subtracting control values.

Measurement of isotope leakage. The petioles of detached leaves were dipped into vials containing a solution of ^{32}P-phosphate ($4\mu c_i$) and incubated at 30C under continuous illumination. After the ^{32}P solution was completely absorbed through the petioles, distilled water was added to the vials. Four hr after ^{32}P treatment, the leaves were cut into halves. One half was inoculated with TMV, another half was served as a non-inoculated control. The leaves were placed in petri dishes, incubated at 22C, and washed with deionized water periodically as described previously. The leaf wash was removed and an aliquot of the solution was placed on a planchet. The planchets were dried, and the radioactivity was determined by using a gas flow counter (Model SCO-5, Aloka Co., Ltd.).

In lesion induction studies, TMV-inoculated plants were incubated at 30C for 2

days, then the inoculated leaves were detached, and ^{32}P solution was absorved through their petioles at 30C. Four hr after ^{32}P treatment, the leaves were cut into halves, subjected to the various temperature treatments as described previously, washed with deionized water, and the radioactivity of the leaf wash was determined. The values were corrected by subtracting control ones. To determine the distribution of ^{32}P in various phosphate fractions from leaf wash and homogenate of leaf samples, inorganic orthophosphate of leaf wash was extracted with isobuthanol as phosphomolybdate [13]. The compounds containing phosphate in leaf samples were fractionated into acid soluble, lipid, nucleic acid and protein fractions by the method of Schneider[28].

The radioactivity of each fraction was determined by a GM counter (Model EDP-101, Aloka Co., Ltd.).

Results

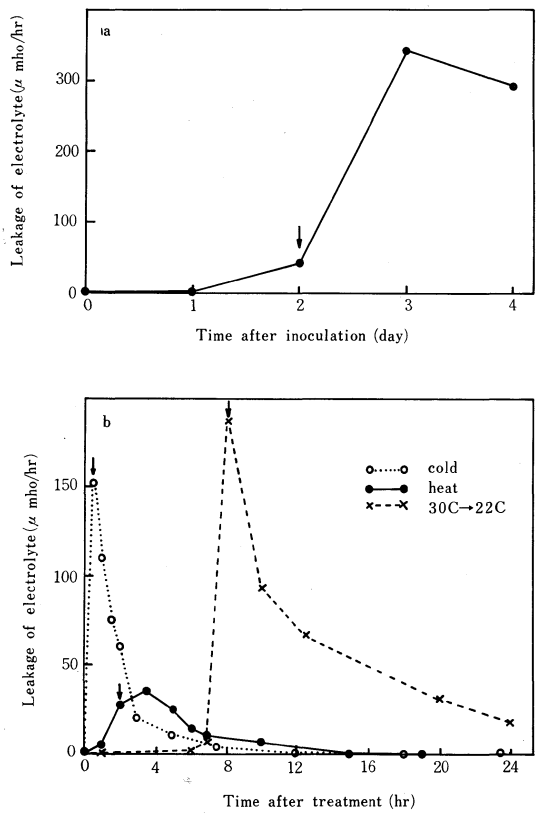

Fig. 2. a) The leakage of electrolytes from *N. glutinosa* tissues after TMV inoculation (constant 22C).
b) The leakage of electrolytes from *N. glutinosa* tissues at various times after the treatments. Tissues treated with: ○···○ ice water (0C, 10 sec), ●—● hot water (50C. 2 min), ×···× transfer to 22C from 30C. The arrow marks the appearance of visible symptoms.

Electrolyte leakage

The results are listed in Fig. 2. When local lesions were normally formed on the inoculated leaves at constant temperature of 22C, electrolyte leakage occurred with the appearance of visible symptoms and its rate increased progressively with the enlargement of the lesions (Fig. 2a).

In heat or cold shock treatments of inoculated leaves, electrolyte leakage occurred rapidly just prior to or during the appearance of visible symptoms (water soaked rings), but its rate decreased with the completion of necrotic lesions. When the leaves were transferred to 22C from 30C, the infected tissues collapsed, induced necrotic rings about 7 to 8 hr later, and the lesions enlarged during subsequent incubation at 22C. In this case, electrolyte leakage occurred rapidly with the appearance of necrotic lesions and its rate further continued during an extended period of incubation (Fig. 2b).

Isotope leakage

The leakage pattern determined with isotope was essentially similar to that determined by conductivity measurement. In all cases, an increase in ^{32}P leakage occurred during the appearance of visible symptoms (Figs. 3a and b). In Fig. 3b, ^{32}P leakage reached a maximum

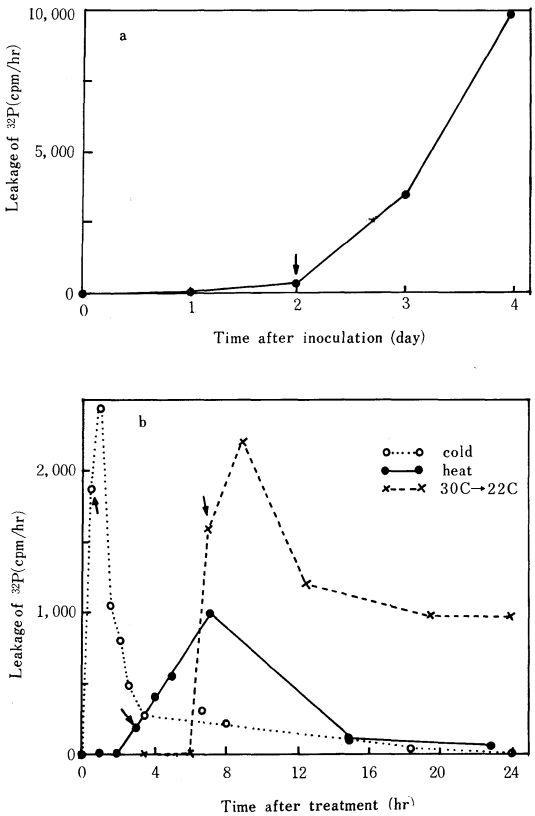

Fig. 3. a) The leakage of ^{32}P from *N. glutinosa* tissues after TMV inoculation (constant 22C).
b) The leakage of ^{32}P from *N. glutinosa* tissues at various times after the treatments noted in Fig. 2b. The arrow marks the appearance of visible symptoms.

Table 1. Distribution of ^{32}P in various phosphate fractions from leaf wash and homogenate of *N. glutinosa* leaf inoculated with TMV[a)]

Sample	Phosphate fraction	Treatments			
		Transfer to 22C		Constant 30C (Control)	
		cpm[b)]	%[c)]	cpm	%
leaf wash	Inorganic phosphate	1,270	1.4	250	0.3
	Organic phosphate	5,000	5.5	30	0.04
Leaf homogenate	Acidsoluble phosphate	63,700	70.3	51,100	63.1
	Lipid	11,300	12.4	14,600	18.0
	Nucleic acid	5,580	6.2	10,900	13.5
	Protein	3,780	4.2	4,140	5.1
Total		90,630	100.0	81,020	100.0

a) After 2 days incubation at 30C, ^{32}P-absorbed leaf half was transferred to 22C and another half was incubated at constant 30C as a control. At 24 hr after the transfer, the radioactivity of various phosphate fractions in leaf wash and in leaf homogenate was determined as described in the text.
b) Figures represent the amount of radioactivity per total quantity of leaf wash or leaf homogenate.
c) ^{32}P absorbed by each fraction expressed as percentages of total quantity.

peak a few hr after the appearance of lesions and its rate subsequently decreased. Table 1 shows the results of experiments on the distribution of ^{32}P in leaf wash and homogenate of *N. glutinosa* leaf, which was kept at 30C for 2 days after inoculation and then transferred to 22C. At 24 hr after the transfer, the amount of ^{32}P contained in each sample was determined. The radioactivity of the wash of leaves transferred to 22C was 7% of that of the ^{32}P initially present in the tissue, and 80% of this activity was detected as organic phosphorus. In contrast, the activity of the leaf wash of control leaves that had been incubated constantly at 30C was much less than that of transferred leaves, and most of this activity was detected as inorganic phosphorus. These results suggest that the radioactivity of the wash of leaves transferred to 22C was probably derived from a loss of intracellular constituents from the tissue in a process of necrosis. Among the various phosphate fractions from homogenate of leaves transferred to 22C, the radioactivity of the acid soluble fraction was slightly greater, and that of the lipid, nucleic acid and protein fractions was less, than that of control leaves that

had been incubated constantly at 30C. These results suggest that the turnover rate of acid soluble fraction into acid insoluble fraction was greater in control leaves than that in transferred leaves.

Discussion

The results of this study indicate that when local lesions are formed on TMV-inoculated *N. glutinosa* leaf, the leakage of cell constituents from the inoculated leaf tissue occurs. Similar leakage was observed when necrotic lesions were induced by various temperature treatments. However, in lesion induction, changes in the leakage of cell constituents was observed within shorter period. Most of the lesions induced by temperature treatments are necrotic rings as described above, and it is postulated that the leakage of cell constituents might occur in these temperature-sensitive rings. It is not clear why the temperature-sensitive zone is confined in the peripheral zone of the areas where the virus has multiplied.

In fungal and bacterial diseases, it has been demonstrated that causal agents produce phospholipase [10, 15, 16, 31] or pectin-degrading enzymes [14, 18, 24, 25]. A possible role of these enzymes was discussed in relation with the pathogenesis. In virus diseases, ectodesmata have been considered to be the portals of entry for viruses[2, 3, 4, 17, 30] and plasmodesmata as pathways for intracellular movoment of virus particles[6, 7, 32]. In this respect, degradation of walls or membranes may not be a prerequisite for viral infection or movement. In the hypersensitive reaction in *N. glutinosa*-TMV combination, however, infected tissues lose their cell constituents, subsequently these areas collapsed to form a necrotic lesion. These facts suggest that the integrity of host cell membranes is altered during virus infection although the nature of the degradation of cell membranes is not clear. A preliminary experiment was done to see whether or not protoplasts isolated from *N. glutinosa* or Samsun NN tobacco by the method of Takebe et al.[1, 29] collapse by the addition of the efflux collected from *N. glutinosa* leaves that had been systemically infected and incubated in dark to induce lesions[23]. *N. glutinosa* or Samsun NN protoplasts treated with lesion efflux did not collapse faster than non-treated protoplasts. Recently, Kato and Misawa[12] reported that deterioration of host cell membranes by lipoxygenase resulted in a local lesion formation on cowpea leaves infected with cucumber mosaic virus.

It may be concluded that a loss of intracellular constituents is an early host response to virus infection in local lesion hosts. However, further investigation is needed to clarify the exact nature of alteration of membrane integrity in cell of infected tissues.

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和文摘要

タバコモザイクウイルスに感染した *Nicotiana glutinosa* 葉での局部病斑形成に伴なう細胞内液の漏出について

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タバコモザイクウイルスの接種により *N. glutinosa* の葉に局部病斑が形成される時，病斑の形成と共に感染部位で細胞内液の漏出がおこることが，電気伝導度の測定または組織に吸わせた^{32}Pの測定によって明らかになった。接種後30Cに2日おいた *N. gluitnosa* の葉（ウイルスは増殖するが無病徴）を22Cに移すか，氷水または50Cの熱水に短時間浸漬する処理によってこの葉に局部病斑類似の病斑が誘導されることを筆者らはすでに報告したが，これらの処理によって病斑が形成される場合にも，病斑の形成にやや先立ってあるいは同時に細胞内液の漏出がおこり，病斑の完成時には漏出が減少していることが判明した。これらの結果は，細胞内液の漏出がウイルス感染による病斑形成の初期過程の1つであることを示しているものと思われる。