

タバコモザイクウイルスおよびサリシレートによるホワイトバーレー葉における β -1,3-グルカンヒドロラーゼ活性の増加とTMVに対する抵抗性の獲得

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Increase of β -1,3-Glucan Hydrolase and Acquisition of Resistance in White Burley Leaves by Tobacco Mosaic Virus and Salicylate

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Abstract

When the leaves of *Nicotiana tabacum* L. cv. White Burley were inoculated with a tomato strain of tobacco mosaic virus (TMV-T), activity of β -1,3-glucan hydrolase was increased, but not with an ordinary strain of TMV (TMV-OM) and mechanical injury. An increase of the enzyme activity in uninoculated upper leaves of the same plants in which the lower leaves were inoculated with TMV-T, was positively correlated with acquired systemic resistance to TMV-T. Both sizes and numbers of local lesions produced on challenge-inoculated upper leaves decreased to about 30% of the control. However, when the inoculated lower leaves were cut off the 2nd day after TMV-T inoculation, no increase of the enzyme activity and systemic resistance in the upper leaves was observed. The enzyme activity was increased when detached half-leaves of the same plant were incubated on sodium salicylate solution, and showed a resistance to inoculated TMV-T. Furthermore, the enzyme activity was increased in the upper leaves of the same plant in which the lower leaves were injected with sodium salicylate and such the upper leaves showed the resistance to TMV-T. Polyacrylamide gel electrophoresis of the enzyme extracts from both the leaves inoculated with TMV-T and the leaves with induced resistance showed the presence of two isozymes of the glucan hydrolase. The involvements of salicylate on the increase of the enzyme activity and acquisition of the resistance to TMV-T are discussed.

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Key words: White Burley, TMV-T, TMV-OM, salicylate, β -1,3-glucan hydrolase, resistance.

INTRODUCTION

The response of tobacco plants infected with virus differs by a combination of host cultivars and strains of virus. *Nicotiana tabacum* L. cv. White Burley is infected systemically by inoculation with an ordinary strain of tobacco mosaic virus (TMV-OM), but the same plant results in necrotic local infection by inoculation with a tomato strain of TMV (TMV-T). The latter phenomenon (so-called hypersensitive reaction) is accompanied by complex biochemical changes that are presumed to strengthen physical and chemical barrier to prevent further infection. *Nicotiana glutinosa* shows the hypersensitive reaction in response to inoculation of TMV-OM. We have reported previously that the activity of β -1,3-glucan hydrolase in *N. glutinosa* increased in the inoculated leaves by infection with TMV-OM⁷⁾ and also in uninoculated upper leaves of the same plant in which the lower leaves were inoculated with TMV-OM⁸⁾. Furthermore, the increase of the enzyme activity in uninoculated upper leaves of *N. glutinosa* occurred coincidentally with the induction of resistance to TMV-OM.

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Recent evidences suggest that salicylic acid is an endogenous signal molecule for inducing pathogenesis-related (PR) proteins, including β -1,3-glucan hydrolase and chitinase in *N. tabacum* L. cv. Samsun NN^{5,6)} and Xanthi-nc^{6,9,11)}. Salicylic acid induced resistance to TMV in Xanthi-nc^{6,10)} and endogenous salicylic acid levels were correlated with accumulation of PR proteins and virus resistance¹²⁾. However, the extent to which PR proteins play a role in salicylic acid-induced resistance is not known.

This paper describes on the increase of the activity of β -1,3-glucan hydrolase in White Burley leaves inoculated with TMV-T and treated with sodium salicylate. The effect of sodium salicylate on resistance to TMV-T was examined.

MATERIALS AND METHODS

Plants and virus. *Nicotiana tabacum* L. cv. White Burley was grown in a greenhouse kept at $25\pm 2^\circ\text{C}$ under natural light. TMV-OM was purified from infected leaves of White Burley leaves according to the method of Gooding and Hebert¹⁾. One g of *N. tabacum* L. cv. Samsun leaves inoculated with a tomato strain of TMV (TMV-T) was homogenized with 10 ml of 0.1 M phosphate buffer, pH 7.0 and the homogenate was centrifuged at $6,000\times g$ for 10 min. The resultant supernatant was diluted with the same buffer to bear 100-400 local lesions per leaf and used as the TMV-T inoculum.

Inoculation with TMV. Fully expanded leaves of White Burley were dusted with Carborundum (400 mesh), inoculating with TMV-OM (25 $\mu\text{g/ml}$), TMV-T or 0.1 M phosphate buffer, pH 7.0. The upper leaves of the same plant in which the lower leaves were inoculated with TMV-T or phosphate buffer, were challenge-inoculated with TMV-T. The size of local lesions appeared on challenge-inoculated upper leaves was measured using a magnifying glass, and the number was also counted the 6th day after challenge-inoculation.

Extraction and determination of β -1,3-glucan hydrolase. Extraction and determination of β -1,3-glucan hydrolase from the leaves were carried out according to the method of Sanada and Matsushita⁷⁾. The enzyme activity was determined as reducing sugars released from water-soluble laminarin (Sigma Chemical Co.).

Treatment with sodium salicylate and inoculation. Healthy detached half-leaves of White Burley were floated on 0.2 mM sodium salicylate solution or distilled water, and incubated in a growth chamber equipped with a cool-white fluorescent lamp (5,000 lux) at 25°C for indicated times. On the other hand, 1 mM sodium salicylate solution or distilled water was injected with a hypodermic syringe into expanded lower leaves of White Burley. At indicated times after incubating or injection, to examine whether the increase of the enzyme activity by salicylate-treatment was corresponded with the induction of resistance to TMV-T, each leaf showing the increase of the enzyme activity was inoculated with TMV-T, respectively.

Electrophoresis. Electrophoresis of the enzyme extracts was performed with 7.5% polyacrylamide gel containing 0.5% water-insoluble laminarin (Biochemicals Co.) according to the method of Sanada and Matsushita⁷⁾. After electrophoresis, the gels were incubated in 0.2 M acetate buffer, pH 5.0, for 60 min at 40°C . Then, the gels were stained with a periodic acid Schiff (PAS) staining procedure.

RESULTS

Activity of β -1,3-glucan hydrolase in the inoculated leaves

Figure 1 shows the time course on the increase of the activity of β -1,3-glucan hydrolase in the leaves of White Burley inoculated with TMV-T. In the leaves inoculated with TMV-T, necrotic local lesions appeared 40 hr after the inoculation and the enzyme activity increased during the hypersensitive reaction. On the other hand, in leaves inoculated with TMV-OM, no increase of the enzyme activity was detected.

Activity of β -1,3-glucan hydrolase in the upper leaves

The enzyme activity in the upper leaves of White Burley in which the lower leaves were inoculated

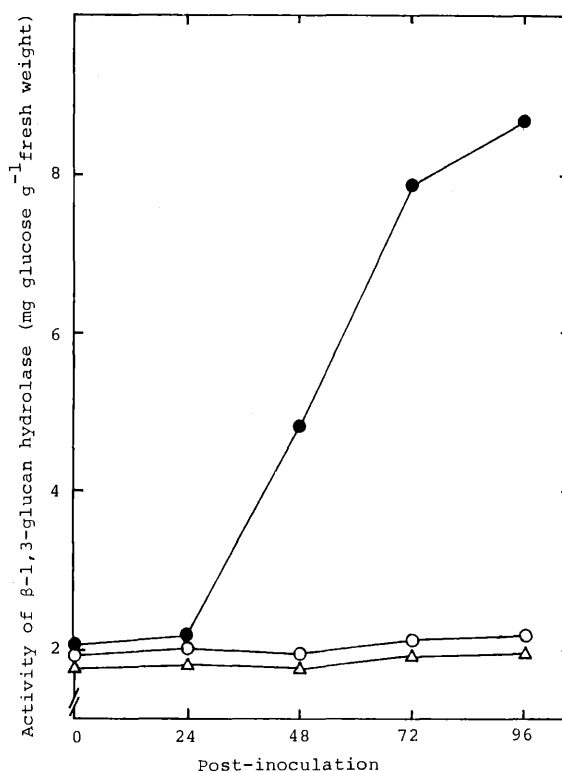


Fig. 1. Activity of β -1,3-glucan hydrolase in White Burley leaves inoculated with TMV. The leaves were inoculated with TMV-T (●), TMV-OM (○) or 0.1 M phosphate buffer, pH 7.0 (Δ). Each value is a mean of three experiments.

with TMV-T increased 4 days after inoculation and reaching about 3-fold values 10 days after the inoculation (Fig. 2).

Acquired systemic resistance

When the upper leaves of White Burley in which the lower leaves were inoculated with TMV-T were challenge-inoculated, acquired systemic resistance to TMV-T was observed. As the result, both sizes and numbers of local lesions appeared on the upper leaves decreased with challenge-inoculated time after the first inoculation of the lower leaves of the same plants and finally resulted to about 30% of the control 10 days post-inoculation (Fig. 2 and Table 1).

Effects of removal of the inoculated lower leaves

When the inoculated lower leaves were cut off the 2nd day after inoculation, no increase of the enzyme activity was detected and no reduction in both sizes and numbers of necrotic lesions appeared in the upper leaves subsequently challenge-inoculated with TMV-T was observed (Table 2). However, the upper leaves showed an increase of the enzyme activity and acquired resistance to TMV-T when the inoculated lower leaves were cut off 4 and later days post-inoculation.

Effect of sodium salicylate on the activity of β -1,3-glucan hydrolase

Figure 3 shows the time course on the increase of the activity of β -1,3-glucan hydrolase in detached half-leaves of White Burley incubated on 0.2 mM sodium salicylate solution at 25°C. As shown in Fig. 3, the increase of the enzyme activity began with the treatment and reached to about 2-fold values of the control 48 hr after the incubation. The chemical increased also the enzyme activity in the upper leaves, although the level of the increase of the enzyme activity (Fig. 4B) was lower than that of the upper leaves of White Burley in which the lower leaves were inoculated with TMV-T (Fig. 2B).

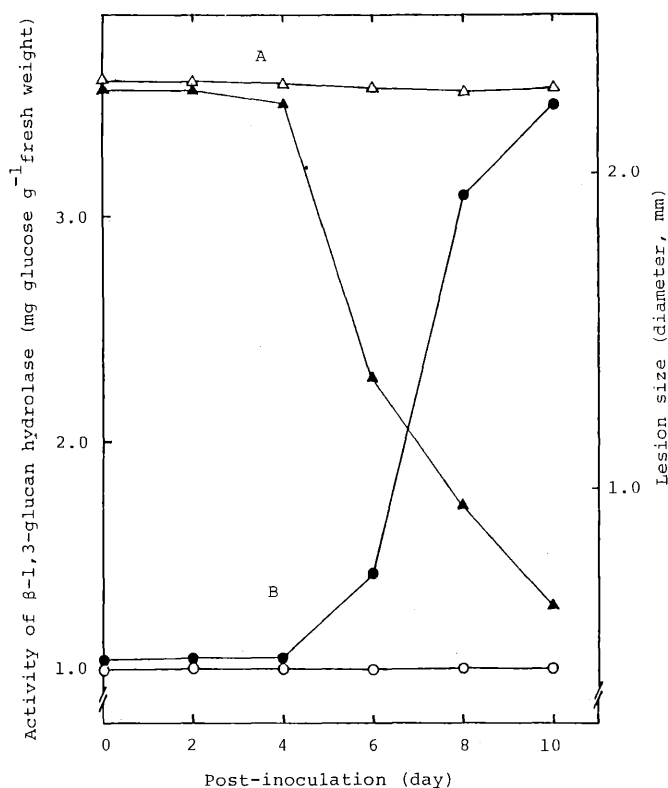


Fig. 2. A : Lesion size on the challenge-inoculated upper leaves of White Burley in which the lower leaves were inoculated with TMV-T (▲) or mock-inoculated with 0.1 M phosphate buffer, pH 7.0 (△). The lesion size was measured 6 days after inoculation. Each value is a mean of three experiments.

B : Activity of β -1,3-glucan hydrolase in uninoculated upper leaves of White Burley in which the lower leaves were inoculated with TMV-T (●) or mock-inoculated with 0.1 M phosphate buffer, pH 7.0 (○). Each value is a mean of three experiments.

Table 1. Number of local lesions produced on the challenge-inoculated upper leaves of White Burley in which the lower leaves were inoculated with TMV-T

Challenge-inoculated time (day)	Number of local lesions/leaf ^{a)}	
	C ^{b)}	T ^{c)}
0	381±21	380±17
2	386±26	375±34
4	353±15	291±14
6	394±24	267±49
8	379±25	188±40
10	396±30	121±16

a) Figures show the mean values and standard deviations in three experiments.

b) C : control plant in which the lower leaves were mock-inoculated with 0.1 M phosphate buffer, pH 7.0.

c) T : test plant in which the lower leaves were inoculated with TMV-T.

Effect of sodium salicylate on resistance to TMV

When detached half-leaves of White Burley incubated on sodium salicylate solution for 24 and 48 hr at 25°C were inoculated with TMV-T, both sizes and numbers of local lesions decreased to about 70%

Table 2. Effect of removal of TMV-T-inoculated leaves on the activity of β -1,3-glucan hydrolase, size and number of local lesions produced on challenge-inoculated upper leaves of White Burley

Time when the inoculated lower leaves were cut off after inoculation (day)	Enzyme activity (mg glucose/g fr.wt.) ^{a)}	Lesion size (diameter, mm) ^{a)}	Lesion number/leaf ^{a)}
2	0.915 $\pm$ 0.073	2.25 $\pm$ 0.01	354 $\pm$ 35
4	1.538 $\pm$ 0.167	2.02 $\pm$ 0.04	194 $\pm$ 16
6	2.165 $\pm$ 0.324	0.97 $\pm$ 0.09	195 $\pm$ 18
8	2.846 $\pm$ 0.146	0.90 $\pm$ 0.09	129 $\pm$ 19
10	3.232 $\pm$ 0.505	0.63 $\pm$ 0.05	121 $\pm$ 16

The upper leaves of White Burley were challenge-inoculated 10 days after inoculation of the lower leaves with TMV-T. Size and number of local lesions produced on challenge-inoculated upper leaves were determined 6 days after challenge-inoculation.

a) Figures show the mean values and standard deviations in three experiments.

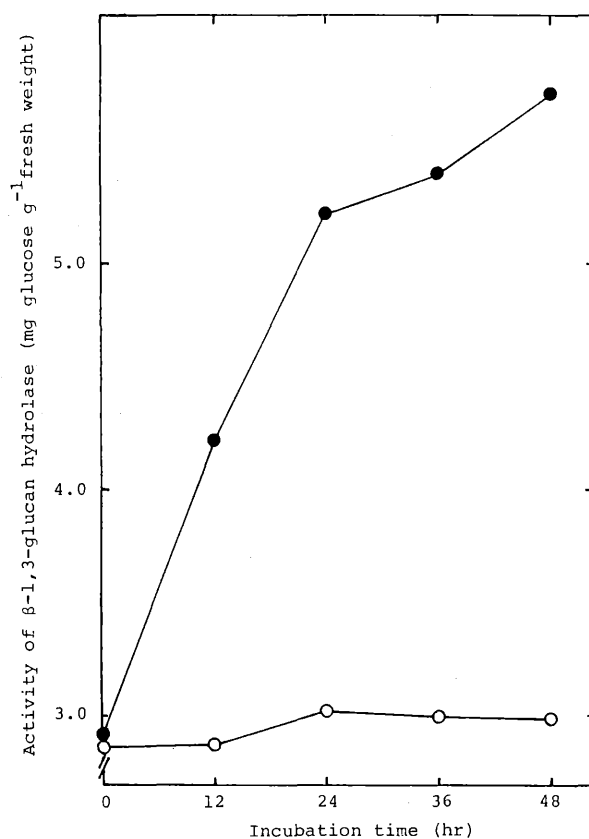


Fig. 3. Activity of β -1,3-glucan hydrolase in detached half-leaves of White Burley incubated on sodium salicylate solution. Detached half-leaves of White Burley were incubated on 0.2 mM sodium salicylate solution (●) and the opposite detached ones were incubated on distilled water (○) at 25°C under continuous illumination. Each value is a mean of three experiments.

and 30% of the control, respectively (Table 3). When the upper leaves of the same plants in which sodium salicylate solution was injected with a hypodermic syringe into the lower leaves were inoculated with TMV-T 8 and 10 days after the injection, the resistance to TMV-T was shown by decrease of both sizes and numbers of lesions (Table 4).

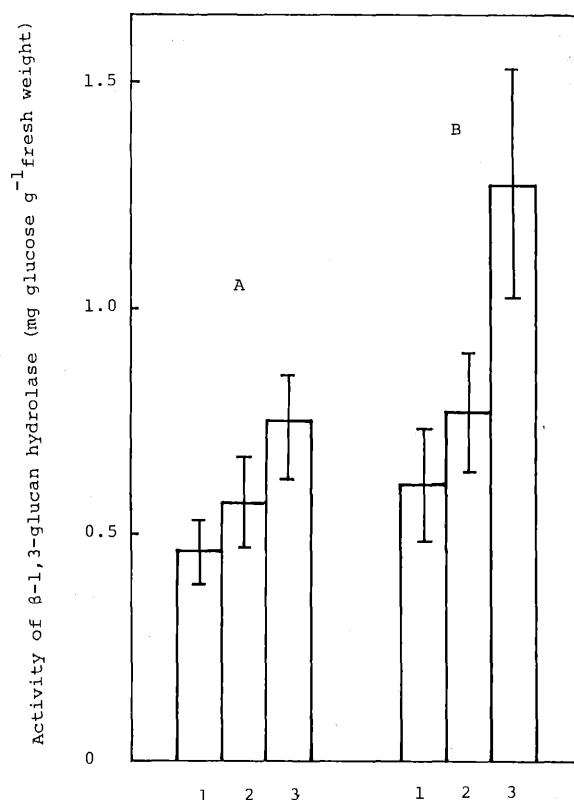


Fig. 4. Activity of β -1,3-glucan hydrolase in the upper leaves of White Burley in which the lower leaves were injected with distilled water (A) or 1 mM sodium salicylate (B) using a hypodermic syringe. The enzyme activity of each leaf-position of the upper leaves was determined the 8th day after the injection. 1 : the top youngest leaves, 2 and 3 : the 2nd and 3rd leaves. Bar shows standard deviation in three experiments.

Table 3. Size and number of local lesions on inoculated detached half-leaves of White Burley incubated on sodium salicylate solution

Inoculated time (hr)	Lesion size (diameter, mm) ^{a)}		Lesion number/half-leaf ^{a)}	
	C	T	C	T
24	2.00 ± 0.03	1.39 ± 0.11	181 ± 13	61 ± 4
48	2.01 ± 0.03	1.41 ± 0.16	172 ± 20	58 ± 9

Inoculation with TMV-T on detached half-leaves of White Burley incubated on distilled water (c) or sodium salicylate solution (T) was carried out 24 and 48 hr after incubation. Size and number of local lesions produced on inoculated detached half-leaves were determined 5 days after inoculation.

a) Figures show the mean values and standard deviations in three experiments.

Electrophoregram

By electrophoretic analysis described in "MATERIALS AND METHODS" only one band of β -1,3-glucan hydrolase was certified in the enzyme extract from healthy leaves of White Burley (Fig. 5a). The band width showing the enzyme activity did not change between those of leaves inoculated with 0.1 M phosphate buffer, pH 7.0, or TMV-OM (Fig. 5b and c). However, the enzyme preparations from the leaves inoculated with TMV-T and from the half-leaves incubated on sodium salicylate showed one and the other additional isozyme bands (Fig. 5d and f). Furthermore, the enzyme preparations from the upper leaves of the same plants in which the lower leaves were inoculated with TMV-T or injected with

Table 4. Size and number of local lesions produced on inoculated upper leaves of White Burley in which the lower leaves were injected with sodium salicylate

Inoculated time (day)	Lesion size (diameter, mm) ^{a)}		Lesion number/half-leaf ^{a)}	
	C	T	C	T
8	1.98±0.02	1.69±0.12	342±34	217±30
10	2.00±0.02	1.42±0.07	365±39	202±25

Inoculation with TMV-T on the upper leaves of White Burley in which the lower leaves were injected with sodium salicylate (T) or distilled water (C) was carried out 8 and 10 days after injection. Size of local lesions produced on inoculated upper leaves was measured and the number was counted 5 days after inoculation.

a) Figures show the mean values and standard deviations in three experiments

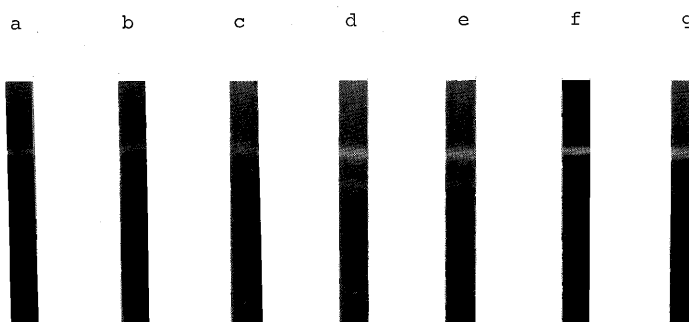


Fig. 5. Electrophoregram of β -1,3-glucan hydrolase of White Burley leaves. a : healthy leaves, b : leaves inoculated with 0.1 M phosphate buffer, pH 7.0, c : TMV-OM (25 μ g/ml)-inoculated leaves, d : TMV-T-inoculated leaves, e : upper leaves in which the lower leaves were inoculated with TMV-T, f : detached half-leaves incubated on 0.2 mM sodium salicylate solution, g : upper leaves in which the lower leaves were injected with 1 mM sodium salicylate. The enzyme activities were determined at the indicated times after inoculation, incubation or injection. Lane a : 0 day ; b,c,d : 4 days ; e : 10 days ; f : 2 days ; g : 10 days.

sodium salicylate solution showed also the same two isozymes pattern (Fig. 5e and g).

DISCUSSION

In White Burley leaves inoculated with TMV-T, the activity of β -1,3-glucan hydrolase increased during the hypersensitive reaction (Fig. 1). The increase of the enzyme activity in inoculated leaves was similar to those reported on *N. glutinosa*^{4,7)} and *N. tabacum* L. cv. Samsun NN²⁾ leaves inoculated with TMV. The enzyme activity did not increase in the leaves of White Burley inoculated with TMV-OM (Figs. 1 and 5c). This fact may be explained by interpretation that an increase of the enzyme activity is closely associated with the formation of local lesions. Furthermore, the increase of the enzyme activity in the upper leaves of White Burley in which the lower leaves were inoculated with TMV-T suggests the presence of systemic resistance to TMV-T (Fig. 2 and Table 1). The enzyme activity of the upper leaves was higher compared with that of the control 8 and 10 days after inoculation which corresponded to the time showing resistance. However, when the lower inoculated leaves were cut off the 2nd day after inoculation, no increase of the enzyme activity in the upper leaves was detected and no resistance to TMV-T was also observed (Table 2). From such the data, it might be suggested that a signal molecule which increase the enzyme activity and the related resistance is transported to the upper leaves from the inoculated lower leaves of White Burley, as suggested in *N. glutinosa* in which the lower leaves were inoculated with TMV-OM⁸⁾.

Salicylic acid induced pathogenesis-related (PR) proteins and resistance to TMV in *N. tabacum* L. cv. Xanthi-nc and Samsun NN^{3,6,10,11)}. In the present study, salicylate-treatment increased the enzyme

activity and induced resistance to TMV-T in detached half-leaves of White Burley (Fig. 3 and Table 3). The chemical increased also systemically the enzyme activity and induced resistance to TMV-T in the same plant (Fig. 4B and Table 4). In the combination with *N. glutinosa* and salicylate, the chemical increased the enzyme activity, but did not induce resistance to inoculation with TMV-OM (data not shown). This may be due to the differences in virus strain or physiological state of the plants.

The results presented in this study support the evidences that salicylic acid is an inducer of β -1,3-glucan hydrolase and resistance to TMV-T also in White Burley, as reported in other cultivars of *N. tabacum* such as Xanthi-nc and Samsun NN^{3,6,10,11}. The role of salicylic acid as a signal molecule in the increase of the enzyme activity and acquisition of resistance to TMV-T has not been fully understood. Combined network of β -1,3-glucan hydrolase (one of the pathogenesis-related proteins) and unknown others may act effects of the virus-resistance.

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和 文 摘 要

真田正幸・伊東理世子・下川英俊・松下亀久：タバコモザイクウイルスおよびサリシレートによるホワイトバーレー葉における β -1,3-グルカンハイドロラーゼ活性の増加とTMVに対する抵抗性の獲得

TMVの普通系でホワイトバーレー葉を接種しても β -1,3-グルカンハイドロラーゼ活性は誘導されないが、TMVのトマト系(TMV-T)では同酵素の活性が誘導された。下葉にTMV-Tを接種した同植物の上葉で検出された同酵素活性の増加はTMV-Tに対する全身的な獲得抵抗性と相関的であった。チャレンジ接種した上葉に形成される局部病斑の大きさとその数は、それぞれコントロール植物の30%に減少した。しかし、下葉を接種後2日目に切除した同植物の上葉では同酵素活性の増加はみられず、またTMV-Tに対する獲得抵抗性も現れなかった。ホワイトバーレーの切り取った半葉をサリシレート溶液(0.2 mM)に浮かべて25°Cでインキュベートすると、 β -1,3-グルカンハイドロラーゼ活性の増加とTMV-Tに対する抵抗性が出現した。さらに、下葉にサリシレート(1 mM)を注入した同植物の上葉でも同酵素活性が増加し、TMV-Tを接種したとき抵抗性が出現した。一方、ポリアクリルアミドゲルによる電気泳動パ

ターンから、TMV-T の接種葉や抵抗性を示す葉には 2 本の同酵素のアイソザイムの存在が確認された。最後に、ホワイトパーレーにおける β -1,3-グルカンハイドロラーゼ活性の増加と抵抗性の誘導に及ぼす TMV-T とサリシレート
の関与及びその意義について考察した。