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Disease Development in Resistant Tobacco Plants Infected with Tobacco Mosaic Virus and Expression of Pathogenesis-Related Genes in Different Temperature Conditions

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TMV에 감염된 저항성 담배 식물체의 온도 조건에 따른 병발생 특성 및 PR 유전자 발현

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ABSTRACT : Tobacco plants resistant (cvs. Xanthi-nc and Samsun-NN) and susceptible (cv. NC 82) to tobacco mosaic virus (TMV) were inoculated with TMV to obtain basic information about the characteristics of resistance expression in tobacco plants by examining the viral populations, symptom development and gene expression of pathogenesis-related proteins (PR-proteins) such as PR-1 and β- 1,3-glucanase in different temperature conditions. TMV populations in resistant plants increased more at 37 ℃ than at 27 ℃, while the viral populations increased continuously and were not significantly influenced by the temperature conditions in the susceptible tobacco plants. Infection sites of resistant tobacco leaves were remarkably expanded in proportion with increased time at the high temperature.

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The PR-1 gene was not induced in resistant tobacco leaves at 37 ℃, irrespective of induction of systemic acquired resistance (SAR) by a prior TMV inoculation, but induced at 27 ℃. Induction of the PR-1 and β-1,3-glucanase genes occurred 6 hours after exposure of the infected plants from the high temperature to room temperature; however, the viral population increased up to 48 hours after exposure to room temperature, suggesting that the PR-protein gene expression may not be related to the inhibition of viral multiplication in infection sites (cells).

Key words: tobacco mosaic virus, resistance, pathogenesis-related proteins, temperature.

The most reliable trait of tobacco resistant to tobacco mosaic virus (TMV) is introgressed from a wild species of tobacco, *Nicotiana glutinosa*. The resistant gene is termed as N gene of which "N" denotes "necrosis", because in resistant plants with the gene is induced hypersensitive response upon TMV infection, forming necrotic local lesions on inoculated leaves. The hypersensitive response triggered by TMV infection is accompanied by numerous metabolic changes related to the resistance to TMV (Bol and Linthorst, 1990).

Van Loon & Van Kammen (1970) and Gianinazzi *et al.* (1970) reported *de novo* synthesis of several proteins, termed as pathogenesis-related (PR) proteins in tobacco plants reacting hypersensitively to infection with TMV. Tobacco plants become resistant to infection from unrelated viruses, fungi, and bacteria after infection with TMV, and moreover induction of PR proteins by abiotic elicitors such as salicylic acid generally results in acquired resistance to pathogens. PR proteins are related to the induction of a broad range of defense reactions (Bol and Linthorst, 1990).

Characteristics and functions of PR proteins have been extensively studied (Antoniw *et al*, 1980; Gianinazzi *et al.*, 1977; Parent and Asselin, 1984; Van Loon, 1976; Van Loon, 1982); however, relatively little attention has been made to the relationships of production of PR proteins with the changes of viral populations in plants, partly because it is difficult to monitor the exact time of the production of PR proteins after TMV infection.

In this study, population changes in susceptible and resistant tobacco plants at different temperatures and expression of PR protein genes such as PR-1 and β-1,3-glucanase in resistant tobacco plants were examined. Also the time of gene expression for PR proteins was monitored to relate it to viral population changes.

MATERIALS AND METHODS

Viral population changes in susceptible and resistant tobacco plants. Tobacco cultivars susceptible *(Nicotiana tabacum* cv. NC 82) and resistant (*N. tabacum* cv. Xanthi-nc) to TMV at about the 10-leaf stage were inoculated by TMV 100 x dilution (w/v, dry weight/buffer of diseased NC 82 tobacco leaf sap in 0.01 M phosphate buffer, pH 7.0) using cotton swap after dusting 600-mesh carborundum. The inoculated plants were placed either at 37 ℃ or 27 ℃ in growth chambers under 12-hour illumination a day. Whole tobacco leaves inoculated with TMV were collected at intervals of one day from 0 to 5 days after inoculation, and ground in 0.01 phosphate buffer with mortar and pestle to obtain 50 x dilution of plant sap. The plant sap of each leaf was inoculated on 2 half-leaves of

Xanthi-nc tobacco. The other half-leaf was inoculated with 50 x dilution of NC 82 tobacco leaf sap with severe mosaic symptoms, and used as control.

Disease development in resistant tobacco plants at high temperature and viral population changes after expression of resistance. Resistant tobacco plants (cv. Xanthi-nc) at the 10-leaf stage were inoculated by TMV as above with the 1,000 x dilution (w/v, dry weight of tobacco leaf/ phosphate buffer) of infected NC 82 tobacco leaf sap, and placed at about 27 ℃ in a greenhouse. Tens of necrotic local lesions were formed 3 days after inoculation, and then the plants were placed at 37 ℃ in the growth chamber for 1 - 4 days. At one-day intervals, plants were exposed to normal temperature (about 27 ℃), and two days later, the expansion of lesion size was measured under a stereomicroscope.

The resistant tobacco plants (cv. Xanthi-nc) were also inoculated with 100 x dilution of the infected tobacco leaf sap as above, placed at 37 ℃ for 2 days, and then exposed to room temperature. Viral population changes in the inoculated tobacco leaves after exposure to room temperature were investigated by the half-leaf inoculation method with three replications as mentioned above.

Total RNA isolation and Northern blot hybridization. Resistant tobacco plants (Xanthi-nc) with or without prior inoculation with TMV were used for total RNA isolation and induction of PR genes by a Northern blot hybridization test. Tobacco plants were inoculated as mentioned above, and placed at high (37℃) and low temperature (27℃) in growth chambers. One, three and/or five days after inoculation, inoculated leaves were collected and used for the extraction of total RNA to assay PR genes. Also inoculated tobacco leaves of resistant tobacco plants (cv. Samsun-NN) incubated at high temperature for 2 days after TMV inoculation and exposed to room temperature were used for isolation of total RNA and Northern blot analysis for PR protein genes.

Total RNA was isolated from frozen leaf tissues by phenol/chloroform extraction followed by LiCl precipitation (Choi et al., 1992). About 20 μg samples of total RNA were separated by electrophoresis through formaldehyde agarose gels. One part of the electrophoresed gel was stained with ethidium bromide and was photographed under UV light to confirm equal sample loading and to detect viral RNA. The other part with same kinds and amounts of samples loaded was blotted to nylon membrane over night, probed with 32P-dCTP labeled PR-1 and β-1,3-glucanase cDNA probes. Hybridization was carried out in 50 % formamide at 42 ℃.

RESULTS

Viral population changes in susceptible and resistant tobacco plants. The TMV population in the susceptible tobacco (NC 82) increased continuously up to 5 days after inoculation, while in the resistant tobacco (Xanthi-nc) it increased initially and then decreased at 5 days after inoculation (Table 1). The maximum viral population was not significantly different in NC 82 between high and low temperatures, but differed significantly in Xanthi-nc. Local necrotic lesions were developed only in Xanthi-nc at the lower temperature 3 days after inoculation; however, no symptoms were observed on the inoculated leaves of the susceptible tobacco and of the resistant tobacco grown at 37℃. At the higher temperature, the initial (1 and 2 days after inoculation) viral population was lower than that at the lower temperature.

Table 1. Viral populations in the susceptible (NC 82) and resistant tobacco (Xanthi-nc) plants grown at high (37℃) and low (27℃) temperatures

Days after inoculation	Relative viral population (%)[a]			
	Susceptible cultivar		Resistant cultivar	
	37℃	27℃	37℃	27℃
1	0.0±0.0	0.0±0.0	1.4±2.2	0.4±0.4
2	0.7±1.6	5.9±2.5	3.1±2.7	17.1±7.1
3	29.6±12.2	8.2±2.7	51.2±11.4	22.6±6.4
5	78.8±10.9	77.5±13.2	34.0±11.8	11.3±2.6

[a] Percentage of viral population relative to that of a susceptible tobacco leaf with severe mosaic symptoms (tested by the half-leaf inoculation method). Numbers are averages and standard deviations of 6 replications.

Table 2. Lesion expansion in the high temperature condition after formation of local lesions by TMV infection

Days at high temperature[a]	Lesion expansion (mm)[b]
0	0.0
1	4.8±1.3
2	9.5±2.5
3	15.2±2.4
4	24.7±5.7

[a] Tobacco plants (cv. Xanthi-nc) with necrotic local lesions on the leaves formed by TMV inoculation (3 days after inoculation) were placed at 37℃.

[b] Distance between the margins of the original and newly formed lesions. Numbers are averages and standard deviations of 25 replications.

Disease development in resistant tobacco plants in the high temperature condition. With the increased period of time at 37 ℃ after formation of necrotic lesions on the inoculated leaves, the necrotic lesions formed by the viral infection (confirmed by exposure to the normal temperature condition, 27 ℃ (Fig. 1)) expanded continuously and more (Table 2). The lesion expansion occurred in all directions except when blocked by thick veins.

Total RNA and induction of PR genes in resistant tobacco plants with or without prior inoculation with TMV. The result of electrophoresis of total RNA in the inoculated leaves, which were maintained at different temperatures after TMV inoculation, is shown in Figure 2. The electrophoretic patterns were similar, and the density of bands were almost identical among the samples, regardless of temperature and time after inoculation, confirming that the RNA amounts loaded for the electrophoresis

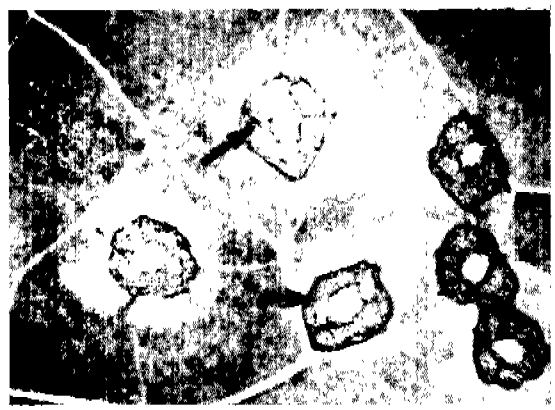

Fig. 1. Expansion of infection sites (outer lesions, arrows) by exposure of the infected leaf of Xanthi-nc tobacco to high temperature (37℃) for 2 days after formation of the necrotic local lesion (inner local lesion). The expanded necrotic lesion was visible 20 hours after the leaf was placed at room temperature (27 ℃)

were not different significantly. Viral RNA was visualized and located at the lowest position of the gel. Strong viral RNA bands were formed on the lanes loaded with samples of high temperature culture for 3 and 5 days, regardless of the 1st and 2nd inoculation tests. Also a weak RNA band was formed on the lane with the 3-day room temperature sample. No or trace bands were formed in other samples, which indicates that the concentration of the viral RNA was lower in the leaf with a prior inoculation on other leaves than in the freshly inoculated leaf at 3 days after inoculation.

The results of the Northern blot test with RNA samples were shown in Figure 3. Of the two PR proteins, PR-1 mRNA was induced remarkably in the resistant tobacco leaves only at room temperature, but not in those at high temperature, regardless of the 1st and 2nd inoculations. On the other hand, β-1,3 glucanase mRNA was produced in all samples, and samples of 1 day after inoculation showed thicker bands than the other samples tested.

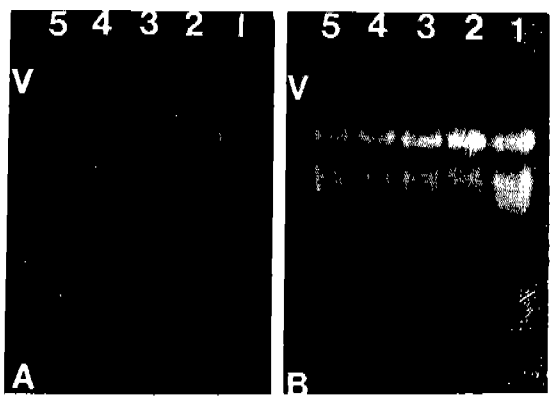

Fig. 2. Electrophoresis of total RNA of tobacco (cv. Xanthi-nc) leaves inoculated with TMV. Xanthi-nc tobacco plants at the 10 leaf stage were inoculated with TMV, and grown at 37℃ (high temperature) and at 27℃ (room temperature). A: 1st inoculation test, B: 2nd inoculation test (plants were reinoculated 20 days after the 1st inoculation). Lanes 1-2: 1- and 3-day growth at 27 ℃; lanes 3-5: 1-, 3- and 5-day growth at 37℃. V: viral RNA.

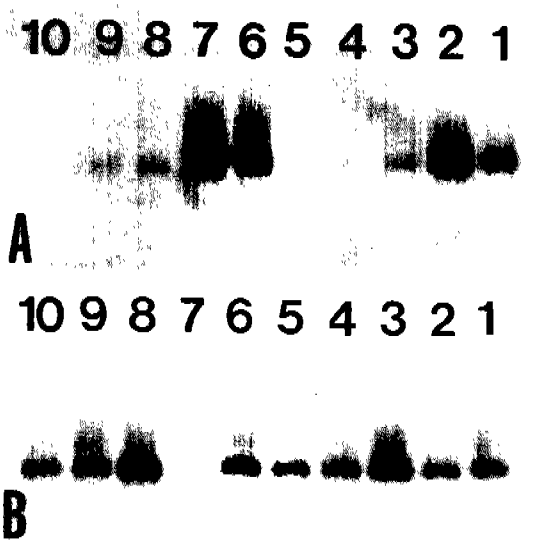

Fig. 3. Northern blot analysis of PR-1 (A) and β-1,3-glucanase genes (B) induced in Xanthi-nc tobacco plant leaves by TMV infection. At the time after inoculation, RNA was extracted from the inoculated leaves (incubated at 37℃ and 27℃), electrophoresed in an agarose gel, blotted onto nitrocellulose and hybridized to ^{32}P-labeled cDNA probes of the PR proteins. Lanes 1-5: 1st inoculation test lanes 1-2: 1- and 3-day growth at 27℃; lanes 3-5: 1-, 3- and 5-day growth at 37℃) Lanes 6-10: 2nd inoculation test (inoculated the upper leaves 20 days after the 1st inoculation) (lanes 6-7: 1- and 3-day growth at 27℃; lanes 8-10: 1-, 3- and 5-day growth at 37℃).

Viral population changes after expression of resistance and induction of PR genes. After resistant plants were placed at 37℃ for 2 days after TMV inoculation, the plants were exposed to room temperature (27℃), and the time of the visible necrotic symptom expression and viral population changes were examined. The original population density of TMV (just at 2 days after inoculation at 37℃) were 17.7 % of the population in the susceptible tobacco with severe mosaic symptoms, slightly increased at 6 hours after exposure to room temperature, and increased greatly 20 - 48 hours later (Table 3). Necrotic local lesions were visible at 20 hours after room temperature exposure, but not up to 6 hours later, indicating that the time of visible symptom expression may be between 6 hours and 20 hours after exposure to the normal temperature conditions.

When the resistant tobacco plants grown at high temperature for 2 days after TMV inoculation were exposed to room temperature, induction of PR genes was detected from 6 hours later (Fig. 4). No significant increase of mRNA for PR proteins was detected up to 3 days after room temperature exposure. This indicates that the resistance response started between 3 and 6 hours after exposure to normal conditions.

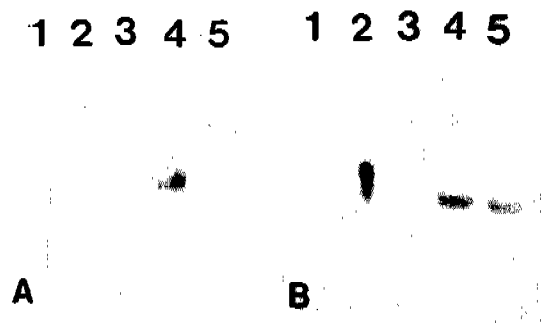

Fig. 4. Northern blot analysis of PR-1 (A) and β-1,3-glucanase genes (B) induced in Samsun-NN tobacco leaves by TMV infection. The infected plants were grown at high temperature for 48 hours and exposed to room temperature. At the time after room temperature exposure (0, 1, 3, 6 and 9 hours: lanes 1-5), RNA was extracted from the inoculated leaves, electrophoresed in an agarose gel, blotted onto nitrocellulose and hybridized to ^{32}P-labeled cDNA probes of the PR proteins.

Table 3. Viral populations and symptom expression in resistant tobacco (Xanthi-nc) plants after exposure to room temper- ature (27℃) conditions

Hours at 27 ℃[a]	TMV population density (%)[b]	Symptom
0	17.7±19.1	-[c]
6	21.6±12.3	-
20	75.3±27.5	Necrosis
48	77.6±39.4	Necrosis

[a] Tobacco plants (cv. Xanthi-nc) grown at 37 ℃ for 2 days were placed at room temperature (27 ℃)

[b] Percentage of viral population relative to that of a susceptible tobacco leaf with severe mosaic symptoms (tested by the half-leaf inoculation method). Numbers are averages and standard deviations of 12 replications.

[c] No visible symptom was observed.

Discussion

The initial viral population was relatively low at high temperature, which may coincide with the fact that TMV infection is inhibited at 38 - 42 ℃ (Lucas, 1975). Thereafter, the viral population in the resistant tobacco increased more at the high temperature than at the low temperature, while in the susceptible tobacco the population increase did

not differ between the different temperature regimes. It is well known that tobacco plants with N gene do not express resistance against TMV infection and are systemically infected when the infected plants are grown in high temperature conditions (over 28 ℃) (Gianinazzi, 1970; Malamy et al., 1992; Westsjeijn, 1984). Necrotic lesions are not formed, replication and spread of the TMV are not restricted, and PR genes are not induced. Yalpani et al. (1991) showed the increase of salicylic acid (which is responsible for the induction of PR-1 genes) was inhibited at high temperatures. Also in our study PR genes were not induced, and infection areas spread with time after local lesion formation in the resistant tobacco in the high temperature conditions.

PR genes were induced within 6 hours after infected plants were shifted from high to room temperature conditions, and visible necrotic symptoms were formed within 20 hours later in this study. Westsjeijn (1981) showed that necrosis ensued only when the low temperature incubation periods exceeded 6 hours. Necrotic lesions appeared 9 to 11 hours after temperature shift from 32℃ to 22℃ (Malamy *et al.*, 1992). These indicate that induction of PR genes are related to the expression of resistance response, and that necrosis formation follows the induction of PR genes. However, viral populations continuously increased up to 48 hours after the temperature shift, suggesting that the virus could multiply after the resistance responses (induction of PR genes and necrosis formation) occurred. These results suggest that the mechanism of resistance to TMV infection may not be related to the inhibition of viral multiplication in an infected area (cell) but inhibition of viral spread from cell to cell.

The hypersensitive necrotic local lesion formation accompanies the increase of the production of oxidases and hydroxyproline-rich proteins that modify the structure of cell wall to form barriers blocking the transmission of pathogens (Bol and Linthorst, 1990). Also formation of cell wall thickenings was suggested to seal-off plasmodesmata though which viruses transmit from cell to cell (Tu & Hiruki, 1971). These aspects support that the resistance mechanism is related to the suppression of cell to cell movement of virions.

Systemic acquired resistance (SAR) is induced when resistant tobacco plants with N gene are inoculated with TMV or treated with SAR inducing agents such as salicylic acid (SA) and its derivatives (Antoniw and White, 1980; White, 1979; Ryals *et al.*, 1994; Yalpani *et al.*, 1994; Malamy and Klessig, 1992; Raskin, 1992; Kessman *et al.*, 1994). However, SAR does not seem to occur in high temperature conditions since no signifiant difference was noted in the viral population between the 1st and 2nd inoculation tests at 37℃ (Fig. 2). Malamy *et al.* (1992) showed that the increases in SA levels were completely inhibited when resistant plants were infected and maintained at 32℃. One of PR proteins, PR-1, which is an indication of SAR, was not induced in the high temperature condition in the resistance-induced plants (Fig. 3). Infection areas spread outside from the necrotic local lesions formed by TMV infection at the room temperature condition (Table 2), in which SAR might be definitely induced. These aspects suggest that SAR is abolished in high temperature conditions.

요 약

담배 모자이크 바이러스 (TMV)에 저항성인 담배 (Xanthi-nc, Samsun-NN)와 감수성인 담배 (NC 82)에 TMV를 접종하고 온도 조건을 달리하여 키웠을 때 담배 잎에서의 TMV 밀도 변화, 병징 발현 특성 및 pathogenesis-related protein (PR 단백질)인 PR-1과 β-1,3-glucanase의 유전자 발현을 조사하여 N gene을

가진 저항성 담배의 저항성 발현 특성을 알아보았다. 저항성 식물체에서 TMV의 밀도 증가는 27 ℃보다 37 ℃에서 더 높았으나, 감수성 식물체에서 바이러스 밀도는 지속적으로 증가하였으며 온도의 영향을 받지 않았다. 감염된 저항성 담배 식물체에서 감염 부위(병반)는 고온에서의 생육기간과 비례하여 현저히 증가하였다. PR-1 mRNA는 전신 획득저항성 유도와 관계없이 고온에서는 유도되지 않았으며, 상온에서 유도되었다. PR 단백질 유전자는 고온에서 48시간 키운 TMV 감염 식물체를 상온에 노출시킨 후 6 시간 이내에 생성되었으나, 바이러스의 밀도는 이 후에도 크게 증가하였다. 이러한 결과는 저항성 담배의 PR 단백질 유전자 발현이 감염 부위 (세포) 내 바이러스 밀도의 억제와 관련되지 않을 가능성을 시사한다.

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