Are 'Pathogenesis-related' Proteins Involved in Acquired Systemic Resistance of Tobacco Plants to Tobacco Mosaic Virus?

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(Accepted 5 October 1981)

SUMMARY

Four host-coded 'pathogenesis-related' proteins accumulate systemically in local-lesion-forming varieties of tobacco after infection with tobacco mosaic virus. It has been suggested that they are involved in the acquired systemic resistance of plants to a second inoculation. Pathogenesis-related protein concentration and amount of resistance (reduction in size and number of lesions formed in the second inoculation) were measured at various times after the first inoculation. The results showed no quantitative or temporal relationship between amounts of resistance and pathogenesis-related proteins. In particular, resistance could be demonstrated in leaves before detectable accumulation of pathogenesis-related protein. Abscisic acid sprayed on plants induced an apparent resistance without inducing pathogenesis-related proteins. Low doses of methyl benzimidazol-2-yl-carbamate caused accumulation of pathogenesis-related protein but not resistance. Nicotiana glutinosa plants accumulated large amounts of a similar protein after infection, but became more susceptible to a second inoculation. All these results suggest that the pathogenesis-related proteins do not play a central role in the mechanism of acquired systemic resistance.

INTRODUCTION

When tobacco cultivars containing the N gene are inoculated with tobacco mosaic virus (TMV), the virus is restricted to the local lesions which form around the infection sites (Holmes, 1938). If half of a lower leaf is inoculated, the uninoculated half and upper leaves become apparently resistant to a subsequent challenge inoculation. This 'induced' or 'acquired systemic resistance' is expressed as a reduction in size and/or number of lesions formed after challenge inoculation (Ross, 1961, 1966; Kassanis et al., 1974; van Loon, 1975).

At least four new host-coded proteins are detected systemically after the first inoculation (Gianinazzi et al., 1970; van Loon & van Kammen, 1970; Kassanis et al., 1974). They are generally absent from healthy plants (van Loon, 1976) and have been referred to as 'pathogenesis-related' proteins (Antoniw et al., 1980). It has been suggested that they may be involved in acquired systemic resistance (van Loon & van Kammen, 1970; van Loon, 1975; Kassanis et al., 1974; Kassanis & White, 1974). However, attempts to demonstrate a direct antiviral effect do not seem to have been successful (Kassanis et al., 1974; Kassanis & White, 1978).

In this paper I report experiments which tested the involvement of the pathogenesis-related proteins in acquired systemic resistance.
**METHODS**

Plants and viruses. *Nicotiana tabacum* L. cv. Xanthi-nc and *N. glutinosa* L. were grown in 12.5 cm diam. pots of Levington compost. Plants which were treated with methyl benzimidazol-2-yl-carbamate (MBC) or their untreated controls were grown in John Innes no. 2 compost. All plants were kept under natural light in a glasshouse, with a minimum temperature of 16 °C and a maximum of 20 to 25 °C.

TMV tomato strain O was multiplied in *N. tabacum* cv. Samsun and purified using the method of Gooding & Hebert (1967). Expanded leaves on 25 cm-tall plants of cv. Xanthi-nc were inoculated by rubbing with a suspension of TMV at 1 to 5 μg/ml 50 mM-sodium phosphate buffer pH 7. Carborundum was used as abrasive; leaves were washed with water after inoculation. There were 10 to 12 leaves per treatment, on 5 or 6 plants. Seven days after inoculation, lesions were counted and the diam. of 60 to 100 lesions per treatment measured using a stereoscopic microscope with ×20 magnification. Differences in lesion size or number between treatments were tested for significance by t-test; lesion numbers were first log-transformed using Kleczkowski's (1949, 1955) transformations.

For measurement of acquired systemic resistance, plants of cv. Xanthi-nc or *N. glutinosa* were inoculated on one-half of each of two lower leaves (the primary inoculation). Control plants were sham-inoculated with buffer and carborundum. At various times afterwards, plants were challenge-inoculated on two upper leaves, and on the previously uninoculated halves of the two lower leaves. Acquired systemic resistance was measured as the percentage reduction in lesion size or number caused by the primary inoculation with TMV.

**Protein extraction and electrophoresis.** For each treatment, leaves were harvested from at least five plants. Samples of 5 g were ground at 0 °C in 5 ml pH 2.8 buffer (84 mM-citric acid; 32 mM-Na₂HPO₄; 14 mM-2-mercaptoethanol) (Antoniw et al., 1980). The homogenate was filtered through muslin and centrifuged at 10,000 g for 20 min. A 5% amount of sucrose (w/v) and a trace of bromophenol blue were added to the supernatant. Samples of 10 to 500 μl of supernatant were fractionated by electrophoresis on 10% polyacrylamide gels (0.67% cross-linked), 75 mm long × 6 mm diam., for 5 h at 3 mA per gel constant current. Buffers were as described by Davis (1964). After electrophoresis, gels were stained overnight in 0.1% PAGE blue G-90 (BDH) in 40% methanol:10% acetic acid, then destained in 14% methanol:10% acetic acid. Destained gels were stored in 7% acetic acid. Mobilities of protein bands (R_f) were expressed relative to that of the bromophenol blue marker. Gels were scanned for absorbance at 260 nm in a Joyce Loebl Gel Scanner; PAGE blue G-90 has an absorption maximum at this wavelength. The peak area of a protein band was shown to be proportional to the weight of protein in the band by electrophoresis of different amounts of the same sample. However, intensity of staining varied from experiment to experiment. To allow comparisons between experiments, samples of a standard extract were fractionated with each batch of gels.

**RESULTS**

**Pathogenesis-related proteins**

Fig. 1 (a) shows electrophoresis of proteins from TMV-infected leaves of cv. Xanthi-nc, with an average of 50 lesions per leaf, extracted 6 days after inoculation. The scan shows four highly mobile bands which are not visible in proteins extracted from comparable, sham-inoculated healthy leaves (Fig. 1 b). The four bands from infected leaves are designated PR1a, PR1b, PR1c and PR2 in order of decreasing mobility, and using the nomenclature system proposed by Antoniw et al. (1980). The R_f values of the bands in 10% gels were 0.86, 0.69, 0.59 and 0.53 respectively. These values correspond well with those reported by Antoniw et al. (1980). PR1a was by far the most prevalent band.
Proteins and TMV resistance

Fig. 1. Polyacrylamide gel electrophoresis of proteins extracted from healthy and TMV-infected plants of *N. tabacum* cv. Xanthi-nc. (a) From leaves bearing an average of 50 lesions, 6 days after inoculation; (b) from comparable sham-inoculated healthy leaves; (c) from healthy leaves on plants sprayed for 14 days with 0.5 mM-ABA; (d) from comparable leaves on control plants sprayed with water; (e, f) from young expanding leaves on healthy plants treated respectively with 2 g or 20 mg MBC; (g) from comparable leaves on untreated control plants. The gels were loaded with protein equivalent to the following fresh wt. of leaf: (a, b) 50 mg; (c, d) 250 mg; (e, f, g) 100 mg.

Fig. 2. Changes in acquired systemic resistance and concentration of PR1a protein with time after inoculation with TMV. At day 0, *N. tabacum* cv. Xanthi-nc plants were inoculated on one-half of each of two lower leaves; these formed 50 to 100 lesions per half-leaf. Control plants were sham-inoculated. The concentration of PR1a protein in infected (■——■) or control (□——□) plants was measured in upper leaves (a) and in the un inoculated half of lower leaves (b). Acquired systemic resistance was measured in upper and lower half-leaves by challenge inoculation at various times and expressed as the percentage reduction in lesion number (●——●) or diameter (○——○) caused by the primary inoculation. For points marked (*) the difference between control and primary inoculated treatments was significant at *P* = 0.05.

The infected leaf protein extract shown in Fig. 1 (a) was used as the standard for calibration of staining intensity in each batch of gels. The concentration of PR1a protein in the standard was defined as 100 units/g fresh wt. Amounts of PR1a protein in other extracts were expressed relative to this. The limit of detection was less than 0.1 unit/g. The four PR proteins were also detected in parts of infected plants which had not been inoculated and which did not develop lesions.
**Time courses of accumulation of pathogenesis-related proteins and development of acquired systemic resistance**

Fig. 2 shows changes in concentration of PR1a in uninoculated parts of the plant with time after inoculation of one-half of each of two lower leaves. Similar changes occurred in PR1b and PR2 concentration. PR1c appeared to show similar changes, although the amount was generally too low for accurate quantification.

In the uninoculated halves of lower inoculated leaves, PR1a became detectable 7 days after the primary inoculation. In uninoculated upper leaves PR1a was first detected 10 days after inoculation of lower leaves. In both cases, the concentration rose to a maximum around 14 to 17 days after inoculation then declined. This interpretation makes the assumption that PR proteins are extracted with the same efficiency as leaves age, an assumption justified by the large amounts of PR proteins recoverable from leaves of senescent, flowering healthy plants (Fraser, 1981).

Upper leaves, and uninoculated halves of lower leaves on both TMV-infected and sham-inoculated control plants were challenge-inoculated with TMV at various times after the first inoculation. The data for percentage acquired resistance are plotted in Fig. 2 at the time of challenge inoculation. Actual lesion sizes and numbers were recorded 7 days later, but differences in lesion size and number between control and 'resistant' plants were clearly established by 3 days after challenge inoculation. The PR protein concentration in the 3 days after challenge inoculation is, therefore, most relevant when investigating the correlation between resistance and PR protein concentration. Control experiments also showed that differences in lesion size and number between treatments were maintained subsequent to the 7th day after challenge inoculation.

Comparing upper leaves and lower opposite half-leaves, it was clear that the amount of resistance induced was generally similar. From 5 to 24 days after primary inoculation, resistance measured as reduction in lesion size was about 40% in upper leaves and 50% in lower leaves. But during this period, the concentration of PR1a in lower half-leaves was around 10 times that in upper leaves.

Within each type of leaf, there was no clear correlation between amount of resistance (measured as reduction in lesion size) and amount of PR protein at various times after primary inoculation. In particular, in lower half-leaves, the resistance remained constant from 5 to 35 days, but PR1a protein concentration varied over a range of more than 30-fold.

In both types of leaf, the curve for resistance (measured as reduction in lesion number) was more similar in shape to the curve for PR1a protein concentration than was the curve for resistance based on lesion size. However, the changes in resistance measured as lesion number tended to develop in advance of the matching changes in PR1a protein concentration, especially in upper leaves. In upper leaves, resistance was detectable when leaves were challenge-inoculated 5 days after primary inoculation; differences in the size and numbers of lesions formed were clearly established by day 9. However, no PR1a protein (or other PR protein) could be detected in this experiment until 10 days after the primary inoculation.

As healthy plants age and begin to flower, they begin to accumulate PR proteins (Fraser, 1981). By 35 days, lower half-leaves on sham-inoculated plants contained similar concentrations of PR1a to comparable leaves on TMV-infected plants (Fig. 2 b). However, the lower half-leaves on infected plants still showed significant resistance, measured on a basis of either lesion size or number.

**Effects of treatment with abscisic acid**

Healthy Xanthi-nc plants were sprayed daily with 0.5 mM-cis-trans-abscisic acid (ABA) for 14 days. Control plants were sprayed with water. After 14 days, proteins were extracted from expanded leaves and similar leaves were inoculated with TMV. The scans (Fig. 1 c
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Table 1. No. and diam. of local lesions formed on plants inoculated with TMV after various pretreatments, and concentration of PR1a protein at the time of inoculation

<table>
<thead>
<tr>
<th>Species</th>
<th>Pretreatment</th>
<th>Lesion diam. (mm)</th>
<th>Lesions/leaf or half-leaf</th>
<th>PR1a concn. (relative units/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. tabacum</td>
<td>Control</td>
<td>2.19 ± 0.07</td>
<td>221 ± 41</td>
<td></td>
</tr>
<tr>
<td>cv. Xanthi-nc</td>
<td>Abscisic acid</td>
<td>1.91 ± 0.07t</td>
<td>66 ± 8t</td>
<td></td>
</tr>
<tr>
<td>N. tabacum</td>
<td>Control</td>
<td>2.04 ± 0.05</td>
<td>23 ± 6</td>
<td>ND~</td>
</tr>
<tr>
<td>cv. Xanthi-nc</td>
<td>MBC (2 mg)</td>
<td>2.01 ± 0.08</td>
<td>25 ± 6</td>
<td>ND</td>
</tr>
<tr>
<td>N. glutinosa</td>
<td>Control</td>
<td>1.27 ± 0.06</td>
<td>60 ± 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pre-inoculated</td>
<td>1.35 ± 0.05</td>
<td>95 ± 12</td>
<td></td>
</tr>
</tbody>
</table>

* N. tabacum plants were given various doses of MBC 9 and 2 days before inoculation, or were sprayed with 0.5 mM-ABA daily for 14 days before inoculation. N. glutinosa plants were inoculated on one-half of two lower leaves; controls were sham-inoculated. Seven days later the opposite halves were challenge-inoculated. All values are means ± standard errors.

† Significantly different from the control at P = 0.05.
ND, Not detectable.

and d) show that ABA treatment did not induce any detectable PR proteins: no bands were seen even in gels very heavily loaded with protein. However, ABA did cause a 70% reduction in lesion number and a small, though statistically significant reduction in lesion size (Table 1). The reduction in lesion size and number, which was found consistently in replicate experiments, contrasts with previous reports of an increase in lesion number or size after ABA treatment at the same concentration (Balazs et al., 1973; Bailiss et al., 1977). The explanation for this apparent discrepancy lies in the different methods used to apply ABA. Balazs et al. (1973) and Bailiss et al. (1977) infiltrated leaf discs with ABA solution. This leads to much larger increases in internal ABA concentration than spraying leaves on intact plants. For example, treatment of leaf discs by infiltration with 50 μM-ABA for 24 h raised internal ABA concentration by around 10 μg/g (Whenham & Fraser, 1980), whereas daily spraying for 8 days with the same concentration raised internal ABA concentration by only 60 ng/g (Whenham & Fraser, 1981 a).

Balazs et al. (1973) and Bailiss et al. (1977) reported visible damage resembling senescence at ABA concentrations which caused increased lesion number and size; the internal ABA concentrations of their leaf discs were undoubtedly far beyond the normal physiological range. In contrast, the ABA-sprayed plants used in this study showed no signs of damage or enhanced senescence, but were slightly darker green than control plants. The increase in their internal ABA concentration was calculated to be around 600 ng/g fresh wt. (Whenham & Fraser, 1981 a) which is within the range of normal physiological concentrations found in tobacco leaves (Whenham & Fraser, 1981 b).

Effects of treatment with MBC

Xanthi-nc plants were treated with various doses of MBC by watering the pots with a suspension of 'Bavistin' (BASF Agrochemicals; 50 % MBC by wt.). In each treatment, plants received two equal doses at day 0 and day 7. At day 9 proteins were extracted and fractionated. Plants treated with a total of 2 mg MBC did not develop detectable PR protein (Table 1). Plants receiving 20 or 200 mg MBC showed detectable amounts of PR1a (Fig 1f; Table 1). The actual amounts of PR1a induced were comparable to those found in upper leaves of TMV-infected plants showing acquired systemic resistance (Fig. 2a). The broad peak of protein with a mobility of 0.75 present in both MBC-treated and control leaves
Fig. 3. Polyacrylamide gel electrophoresis of proteins extracted from healthy and TMV-infected
*N. glutinosa* plants. (a) Extracted from uninoculated halves of leaves 7 days after inoculation of
the opposite halves; (b) from comparable leaves on sham-inoculated control plants. Gels were
loaded with protein equivalent to 100 mg fresh wt. of leaf.

(Fig. 1f, g) was a host protein characteristic of younger leaves; it disappeared as the leaf
completed expansion (Fig. 1b).

The PR1a protein induced by 200 mg MBC was partially purified by gel-filtration and
ion-exchange chromatography (Antoniw et al., 1980) as described earlier (Fraser, 1981)
and was found to co-migrate with authentic, TMV-induced PR1a protein when fractionated
by electrophoresis in non-denaturing and denaturing (SDS) gels (data not shown). Thus, there
can be little doubt that the protein induced by MBC was the same as the PR1a induced by
TMV.

MBC doses of 20 or 200 mg/plant did not induce detectable amounts of the three minor
PR proteins Ib, lc and 2. Plants treated with 2 g MBC had high concentrations of PR1a
and also detectable PR1b, lc and 2 (Table 1, Fig. 1e). Nine days after the first MBC
treatment, comparable leaves on further plants were inoculated with TMV; lesion size and
number were subsequently measured. Treatment with MBC at up to 200 mg per plant caused
no significant change in size or number of lesions, although plants treated with 20 or 200 mg
MBC did contain detectable PR1a protein (Table 1). Plants treated with 2 g MBC formed
significantly fewer and smaller lesions than the controls. However, 2 g MBC also caused
direct phytotoxic effects by 9 days, including reduced growth, yellowing and necrotic spots on
uninfected plants. The effect on lesion development may, therefore, have been a result of
this phytotoxicity.

*A pathogenesis-related protein in N. glutinosa*

Leaves on *N. glutinosa* plants were inoculated on one-half with TMV and formed an
average of 50 lesions. Seven days later, proteins were extracted from the uninoculated
halves. Comparable uninoculated halves were challenge-inoculated. Fig. 3 shows that TMV
infection caused accumulation of very large amounts of a protein with mobility 0.74 in the
uninoculated half of the leaf. Van Loon & van Kammen (1970) also found a band of similar
mobility. Fig. 3 shows that the *N. glutinosa* protein is similar to the PR proteins of *N.
tabacum* in that it is highly soluble at pH 2.8.
Table 1 shows that half-leaves opposite previously inoculated halves appeared to form more lesions than the controls when challenge-inoculated. In replicate experiments, the primary inoculated plants formed 1.2 to 1.7 times as many lesions as the controls. There was no difference in lesion size. Upper leaves of TMV-infected *N. glutinosa* plants also contained the PR-like protein and formed significantly more lesions after challenge-inoculation than control plants; lesion size on upper leaves was unaltered or very slightly reduced as a result of prior inoculation of lower leaves (Fraser *et al.*, 1979).

**DISCUSSION**

The four PR proteins have been shown to occur in leaves which also show acquired resistance as a consequence of localized TMV infection elsewhere in the plant (Kassanis *et al.*, 1974; van Loon, 1975; Rohloff & Lerch, 1977). Several experimental treatments applied to healthy plants will also induce PR proteins and resistance to TMV. These include polyacrylic acid (Gianinazzi & Kassanis, 1974), acetylsalicylic acid (White, 1979) and plant hormones (Antoniw *et al.*, 1981). Thus, the evidence for involvement of PR proteins in acquired systemic resistance is comprehensive but entirely correlative.

In this paper, PR protein concentration and amount of resistance were measured at various times after the primary inoculation, to test further the correlation between the proteins and resistance. The results suggest that there is not a close quantitative relationship between the amount of PR protein present and the amount of acquired systemic resistance. Furthermore, examination of the temporal relationship between changes in PR protein concentration and amounts of resistance also provided evidence against a role of the proteins in resistance. In particular, resistance could be demonstrated in leaves before detectable accumulation of PR protein occurred.

The second experimental approach was to attempt to induce PR proteins and resistance independently of each other. ABA induced an apparent resistance, measured as a reduction in lesion number, without inducing detectable PR proteins. ABA had, however, only a small effect on lesion size. Treatment with low doses of MBC induced accumulation of significant amounts of PR1α protein without inducing any resistance, although higher, phytotoxic doses of MBC induced both proteins and apparent resistance. Finally, *N. glutinosa* was shown to accumulate large amounts of a protein similar to the *N. tabacum* PR protein after TMV infection. But in *N. glutinosa*, this protein was associated with an increase in susceptibility to infection and not with resistance. Thus, these experiments indicated that it was possible to induce PR protein without resistance and that effects similar to resistance could be induced without induction of PR protein.

A necessary qualification to the experiments with chemical induction of PR proteins or apparent resistance is that the mechanisms involved might not be the same as with virus as inducer. This does not, however, exclude them as reasonable experimental tests. Are the PR proteins therefore involved in acquired systemic resistance? Logically, it is impossible to prove the negative statement that PR proteins are not involved. Any model relating proteins to resistance can theoretically be modified to accommodate additional experimental evidence. However, the different types of experimental test described in this paper all suggest that the PR proteins are not centrally involved in acquired systemic resistance. This suggestion raises two further questions: what is the function of PR proteins if not resistance, and, what is the mechanism of the apparent systemic resistance? The wide variety of factors which will induce PR proteins: viral infection (Gianinazzi *et al.*, 1970; van Loon & van Kammen, 1970); fungal infection (Gianinazzi *et al.*, 1980); various chemical treatments (Gianinazzi & Kassanis, 1974; White, 1979; Antoniw *et al.*, 1980) and flowering of the plant (Fraser, 1981) suggests that the PR proteins are some general response to stress conditions. The two aspects of acquired systemic resistance, reduction in lesion size and
number, seem to involve separate mechanisms, as they can apparently operate to some extent independently (e.g. Fig. 2, Table 1 and Fraser et al., 1979).

Alteration in lesion number does show an inverse correlation with changes in ABA concentration. In *N. glutinosa*, which shows an increase in lesion number as a result of the first inoculation, ABA concentration in the challenge inoculated leaves is reduced (Fraser et al., 1979). In *N. tabacum*, where lesion number is reduced as a result of the first inoculation, ABA concentration of the challenge-inoculated leaves is increased by the primary inoculation (Whenham & Fraser, 1981a). The reduction in number of lesions formed when healthy plants were sprayed with ABA before inoculation is also consistent with this correlation. Whether ABA is the direct cause of altered lesion formation, or indirectly involved through an effect on leaf water status and mechanical susceptibility to inoculation (Cassells et al., 1978) remains to be seen.

Reduction in lesion size could involve actual inhibition of virus multiplication, or merely a retardation of necrotization. Evidence that virus multiplication is reduced in acquired systemic resistance is conflicting (Ross, 1966; van Loon & Dijkstra, 1976; Balazs et al., 1977; Fraser, 1979) and the topic requires further study.

I thank Su Loughlin for capable assistance.

REFERENCES


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(Received 18 May 1981)