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Pathogenesis-related proteins and acquired systemic resistance: causal relationship or separate effects?

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Abstract

This paper questions whether pathogenesis-related proteins (PRs) have any role in acquired systemic resistance, and whether there may be alternative explanations for the reduced number and size of lesions formed when leaves containing PRs are inoculated with virus. It is concluded that PRs may not play a direct role in acquired resistance; that altered lesion number may result from altered susceptibility of the leaf to mechanical inoculation, and that reduced lesion size could reflect a non-specific modulation of the basic localization mechanism. Preliminary experiments showing changes in ultrastructure of leaves associated with the development of acquired systemic resistance are discussed. The most striking change was development of myelinic bodies, generally between the cell wall and plasmalemma in uninoculated areas of leaf opposite halves bearing lesions.

Additional keywords: Nicotiana tabacum, N-gene, hypersensitivity, local lesions, virus localization, electron microscopy, myelinic bodies.

Introduction

When plants form necrotic lesions after inoculation with virus, the response of uninfected parts of the plant to a second ('challenge') inoculation can be altered. Lesions formed after challenge inoculation may be smaller or less numerous than those formed on previously-uninoculated control plants. This 'induced' or 'acquired systemic resistance' (ASR) has been found in several host-virus combinations (Ross, 1961).

In tobacco cultivars which form lesions after infection with tobacco mosaic virus (TMV), up to ten new host-coded proteins are detectable in parts of the plant showing ASR (Gianinazzi et al., 1970; Van Loon and Van Kammen, 1970; Van Loon, 1982). It has been suggested that these 'pathogenesis-related' proteins (PRs) may be involved in ASR, perhaps in a way analogous to interferon in animal cells (Kassanis et al., 1974; Gianinazzi, 1982). Proteins with properties similar to those of the tobacco PRs have been found after necrotic infection of other hosts (e.g. Coutts, 1978).

Several experimental treatments will induce ASR and PRs in previously uninoculated plants. These include polyacrylic acid (Gianinazzi and Kassanis, 1974); acetyl salicylic acid (White, 1979) and plant hormones (Antoniw et al., 1981). Thus the evidence for the involvement of PRs in ASR is comprehensive but entirely correlative. However, some authors have questioned whether PRs are actually involved in ASR (Fraser, 1981, 1982a, 1982b), or have reported experimental situations where some evidence does not support involvement (Van Loon and Antoniw, 1982).

Our objective in this paper is to assess whether PRs are involved in ASR, and to evaluate other possible explanations of the two effects. The following questions may be raised; some have yet to be fully tested.

1. Are PRs involved in ASR and if so, what is their mode of action?
2. Are PRs an integral and essential part of the localization of the primary inoculation, or are they purely associated with modification of the second infection?
3. If PRs are not involved in ASR, what are the mechanisms involved in controlling the observed reductions in lesion size and number?
4. If PRs are not involved in resistance, what do they do?
5. Is ASR actually a resistance mechanism, or does it merely look like one?
6. Is there any prospect for exploitation of ASR in practical crop protection?

In this paper we review the available evidence on the first three of these questions. We present a preliminary report on studies of ultrastructural changes during induction of PRs and ASR, which may have a bearing on the fourth question. We conclude with a general consideration of questions 5 and 6.

Are PRs involved in acquired systemic resistance?

If PRs are involved in ASR, the following predictions may be made:

1. The amount of resistance would show some temporal and quantitative relationship to PR protein concentration.
2. ASR would not occur in the absence of PRs.
3. PRs would not occur in the absence of ASR.
4. The proteins would have some demonstrable role in the mechanism of ASR.

If any of these predictions is shown experimentally to be invalid, this does not necessarily prove that PRs have no role in ASR. Indeed, it is logically impossible to prove that the proteins are not involved in resistance, because theoretically the model relating them to resistance can always be modified to account for new experimental data. However, models which become festooned with conditions become inherently less likely. A demonstration that any or all of the above predictions is invalid would weaken the case for involvement of PRs in ASR. We will now summarise evidence against the predictions.

1. The quantitative or temporal correlations between the concentrations of PRs (although only a limited number have been measured) and the amount of ASR are not good. In 'Xanthi-nc' tobacco, ASR could be detected in half leaves opposite previously inoculated halves, or in previously untreated upper leaves, before PRs could be detected (Fraser, 1982a). The concentration of PR 1a in opposite half leaves and upper leaves rose, then declined rapidly with time after inoculation. The time course for ASR expressed as reduction in lesion diameter bore no relationship to the curve for PR 1a concentration. The time course for ASR expressed as reduction in lesion size was similar to the curve for PR 1a concentration, but preceeded it. Upper leaves accumulated only about one tenth as much PR 1a as lower, opposite half leaves, but the amount of ASR in the two locations was comparable.

Healthy plants accumulate PRs as they enter the flowering phase. Removal of either or both of the developing inflorescence and the senescing lower leaves can reduce PR accumulation by up to 90%. When healthy plants were variously treated to create a

range of PR concentrations and then inoculated with TMV, there was no correlation between the sizes and numbers of lesions formed and PR concentration (Fraser, 1981).

All these data therefore argue against any close temporal or quantitative relationship between PR concentration and amount of ASR.

2. Some chemical treatments may induce ASR-like effects in the absence of detectable concentrations of PRs. Spraying 'Xanthi-nc' plants with low doses of abscisic acid (ABA) significantly reduced both the size and numbers of lesions formed on subsequent inoculation, without inducing PRs (Fraser, 1982a). Treatment of 'Samsun NN' tobacco plants by injection of salicylic acid into leaves caused accumulation of PRs and ASR. Untreated leaves on these plants did not accumulate PRs, but did often display ASR, measured as reduction in lesion size (Van Loon and Antoniw, 1982).

3. Treatment of 'Xanthi-nc' plants with low doses of methyl benzimidazol-2yl-carbamate caused accumulation of some PR protein, but no detectable ASR (Fraser, 1982a). In *N. glutinosa* plants large amounts of a PR-like protein accumulated after inoculation. Lesions formed on such plants after a challenge inoculation were slightly smaller than those on control plants, but lesion number was actually increased as a consequence of the first inoculation (Fraser et al., 1979).

Together, these three types of correlative evidence argue against a role of PRs in ASR.

4. The strongest type of evidence for involvement of PRs in ASR would be demonstration of a function in the resistance mechanism. At the time of writing no such evidence is available. When purified PRs were added to protoplasts, they caused no reduction in TMV multiplication (Kassanis and White, 1978).

A demonstrated role of PRs in a biological process not directly related to resistance would be good evidence, though not absolute proof, that PRs have no part to play in ASR. At present there is no alternative function for PRs which is supported by experimental evidence. Increasing knowledge of the chemistry and metabolism of PRs (c.f. Carr et al., 1982) may eventually suggest possible functions.

Are PRs an integral part of the localization response to the primary inoculation?

Although PRs accumulate to high concentrations in primarily-inoculated leaves (Van Loon and Van Kammen, 1970), there has been surprisingly little work on whether they are involved in the primary localization of virus. Sela (1981) hypothesized that PRs might be the means by which the putative antiviral protein AVF confers resistance on uninfected cells: at present there is no experimental evidence for this suggestion. Van Loon and Van Kammen (1970) reported that PRs appeared in 'Samsun NN' leaves 3 to 4 days after inoculation, at the onset of necrotisation. It is likely that by this time, considerable restriction of virus spread had already occurred (c.f. Loebenstein et al., 1982).

When 'Samsun NN' plants were grown at 30 °C TMV spread systemically, and no PRs accumulated (Van Loon, 1975). Plants grown at 20 °C localized the virus and accumulated PRs. When such plants were transferred to 30 °C, the virus spread systemically, despite the continued presence of PRs.

These lines of evidence therefore suggest that PRs might not be directly involved in

the primary localization of virus. However, the primary localization may offer useful and unexploited situations for testing the metabolic and biological properties of PRs.

Are other mechanisms possibly involved in the control of altered lesion size and number?

There is evidence that the two components of ASR: reduction in lesion size and reduction in lesion number, may be independent, and under separate controls. In 'Xanthinc' plants, the time courses of ASR expressed either as reduced lesion size or number were quite different (Fraser, 1982a). In *N. glutinosa,* a previous inoculation of lower leaves caused slightly reduced lesion size when upper leaves were challenge-inoculated, but a simultaneous increase in lesion number (Fraser et al., 1979).

The evidence suggests that alteration in lesion numbers might reflect a change in the susceptibility of the leaf to mechanical wounding. At least part of this may be due to altered leaf water status: turgid leaves are thought to be more susceptible to wounding than flaccid ones (Yarwood, 1959). Cassells et al. (1978) showed that spraying plants with an anti-transpirant abolished the reduction in lesion numbers induced by polyacrylic acid treatment. An inverse correlation between increase or decrease in lesion numbers, and changes in leaf concentration of ABA was reported by Fraser et al. (1979), Fraser (1979) and Whenham and Fraser (1981). ABA is an indicator of a leaf's history of water stress. This work also showed that sufficient ABA was transported from the lower, primarily inoculated leaves to the upper leaves in 'White Burley' tobacco to explain the increased ABA concentration of the upper leaves, and the reduced numbers of lesions formed on challenge inoculation.

The reduction in lesion size in ASR may (Ross, 1966; Van Loon and Dijkstra, 1976) or may not (Balazs et al., 1977; Fraser, 1979) be associated with a reduction in virus multiplication. Unfortunately, all these reports used different host-virus combinations, and there is a clear need for further study of the effects of ASR on virus multiplication.

It has been suggested that reduction in lesion size could reflect mainly a reduction in necrosis (Balazs et al., 1977) as a result of virus-stimulated increase in leaf cytokinin concentration. However, the demonstrated changes in cytokinin were small, and the effects of exogenous cytokinins on lesion development are so variable (reviewed in Fraser and Whenham, 1982) as to make interpretation of these experiments difficult.

Early in the localization process, there is an increase in phenylpropanoid synthesis which is doubtless involved in necrogenesis and may be involved in virus localisation (Simons & Ross, 1966; Fritig et al., 1973; Legrand et al., 1976). There is some, though still inconclusive evidence that leaves with ASR show more rapid activation of the phenylpropanoid pathway after challenge inoculation than control leaves (reviewed by Van Loon, 1982). Legrand et al. (1978) have suggested that the synthesis of lignin using precursors from this pathway could limit lesion spread.

Ultrastructural changes associated with acquired systemic resistance: a preliminary study

The new proteins synthesised in large amounts in association with the acquisition of systemic resistance share certain common biochemical features and might arguably

have a structural rather than a catalytic role. They are found in the cell wall and/or intercellular spaces, (L.C. van Loon, personal communication) and the spread of virus and/or necrosis from cell to cell is normally inhibited in leaves containing these proteins, as is shown by smaller lesions. It was therefore interesting to examine whether the occurrence of PRs and ASR was accompanied by changes in cell wall ultrastructure or synthesis.

Figures 1-3 show multi-lamellar bodies found in the uninoculated halves of leaves bearing lesions on the other halves. These bodies were composed of concentric layers of membranes and were similar in appearance to the myelin sheath surrounding animal nerve fibres. They will be referred to as myelinic bodies. They were generally located between the cell wall and the plasma membrane: Fig. 2 shows the membrane curving inside the myelinic body, although the inner part of the body may have been inserted into the cytoplasm. Myelinic bodies were not generally found sandwiched between the cell wall and chloroplasts, but were appressed to areas of cell wall between adjacent chloroplasts or mitochondria (Fig. 1). Myelinic bodies which were not clearly associated with the cell wall were detected very rarely.

The myelinic bodies were complex structures of multilamellar membranes and enclosed spaces, and clearly had an intricate three-dimensional structure. The enlarged section in Fig. 3 shows the multiple-membrane structure cut in transverse section, and also some faces of roughly granular material, presumably the same membranes seen in surface view.

An equal time was spent examining sections of control material. Fig. 4 shows an example of a loosely-aggregated membrane body associated with the cell wall in control material. Such structures obviously were much less organised than the myelinic bodies associated with infection, and were also infrequent. Only one small myelinic body was seen in all the control material examined.

Uninfected halves of previously infected leaves also showed an increased occurrence of looser membrane structures such as shown in Fig. 4, as well as cytoplasmic structures similar to lomasomes or prolamellar bodies. These have not yet been examined in detail.

Various membranous organelles: the Golgi apparatus, lomasomes and paramural bodies, are thought to be involved in cell wall synthesis, elaboration and materials transport (Gardiner and Chrispeels, 1975; Bowes, 1979; Kawasaki, 1981). Several types of virus infection modify cell walls and related ultrastructure. Paramural bodies and extended, bulbous plasmodesmata accompanied infection of oat leaves with barley stripe mosaic virus (McMullen et al., 1977) and bean leaves with bean pod mottle virus (Kim and Fulton, 1973). Paramural bodies were also found at the periphery of TMV lesions on 'Pinto' beans (Spencer and Kimmins, 1971). In tobacco leaves, vesiculate membranes appressed to cell walls (plasmalemmasomes) and microvesicles were sometimes found in tissue adjacent to TMV lesions (Simons et al., 1972) but were more common when the leaf was heated (Ross and Israel, 1970). In contrast to the previous cases where paramural bodies were thought to be involved in cell wall elaboration, the tobacco leaf organelles were considered to be related to incipient cell collapse.

All of these reported membrane structures were considerably looser and less organised than the myelinic bodies we report here. Kim et al., (1974) found myelinic bodies which partly resembled the tobacco bodies, in leaves infected with two comoviruses. As with tobacco, the bean leaf bodies were located between the cell wall

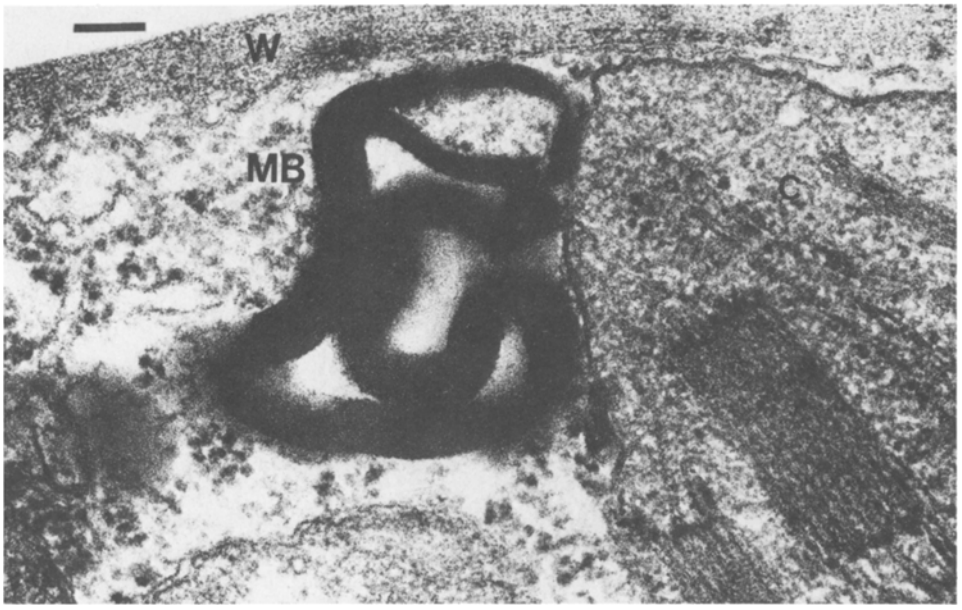
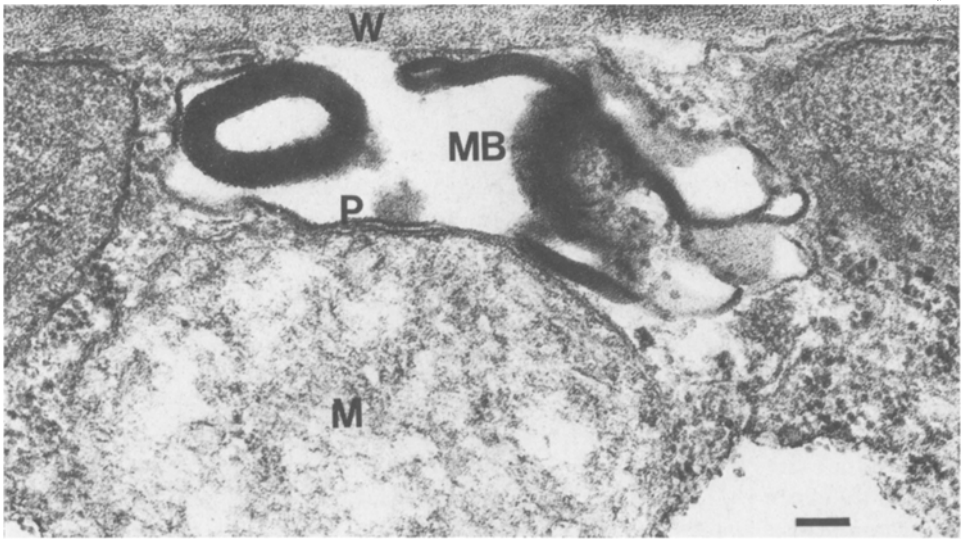


Fig. 1

Fig. 2



Figures 1-4. Ultrathin sections of tobacco cv. Xanthi-nc-leaf. Figs 1-3: tissue from opposite a half leaf which had formed lesions after inoculation with TMV. Fig. 3 shows an area of Fig. 2 at higher magnification. Fig. 4: tissue from a healthy control leaf. The bar in each plate represents 100 nm. C, chloroplast; W, cell wall; P, plasmalemma; M. mitochondrion; MB, myelinic body; PB, prolamellar body.

'Xanthi-nc' tobacco plants 20 cm-tall were inoculated with TMV on one half of two expanded leaves, and developed on average 100-200 lesions per half leaf. By seven days, the inoculated leaves showed PRs and ASR in their untreated halves (cf. Fraser, 1982a).

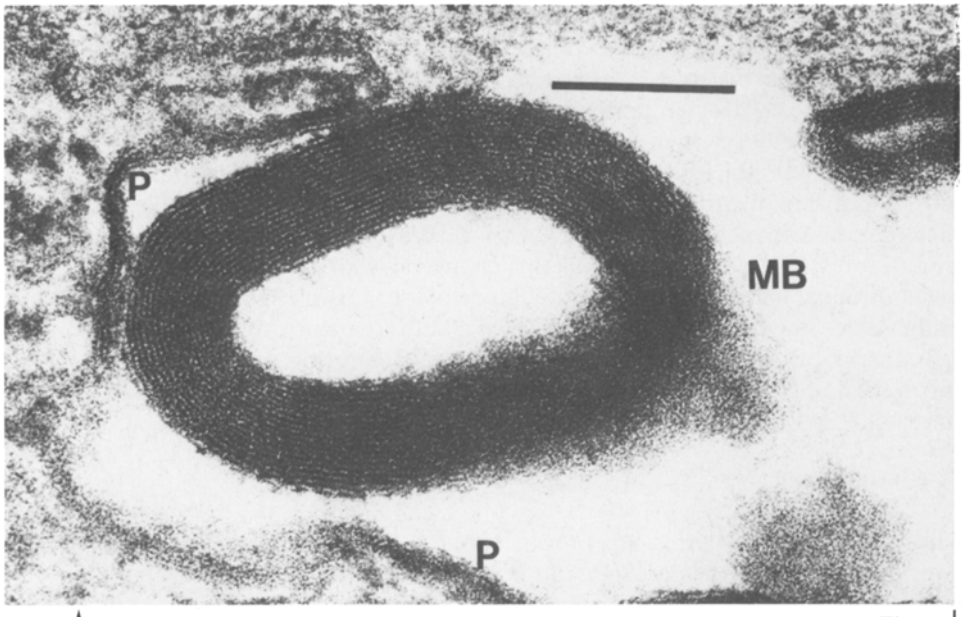
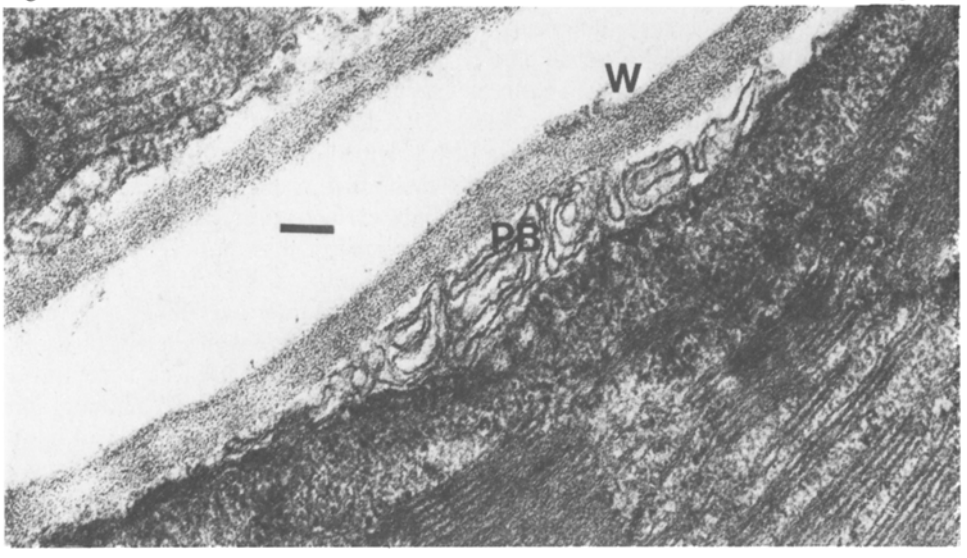


Fig. 3

Fig. 4



Triangles of leaf (5 × 15 × 15 mm) were cut from the interveinal area of untreated halves of infected leaves or from comparable areas of control leaves, fixed in 2% (v/v) glutaraldehyde in 0,1 M sodium cacodylate buffer, pH 7,2, at 20 °C, and rinsed for 30 min in the same buffer. After treatment with 1% (w/v) osmium tetroxide in cacodylate buffer for 2 h at 20 °C, samples were rinsed again in buffer for 30 min.

Samples were dehydrated through an ethanol series and embedded in L.R. White Resin (medium grade) (London Resin Co.). Ultrathin sections (approximately 50-100 nm thick) were cut on a Reichart Ultracut ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a JEOL 100CX II transmission electron microscope.

and deeply invaginated plasmalemma. However, the bean leaf bodies appeared to have fewer enclosed spaces, and a less complex three-dimensional structure than the tobacco leaf bodies. Furthermore, the bean leaf bodies were in virus-infected cells, and were often associated with membranes of tubules containing virus particles, whereas the tobacco leaf bodies were in tissue 10-50 mm distant from the infection.

What (if any) is the functional significance of the myelinic bodies, and do they have any relationship to ASR or the PRs? It is possible that the myelinic bodies and other membranous organelles are involved with increased cell wall synthesis or modification. At present, we are studying whether changes in wall ultrastructure occur in tissue when myelinic bodies (and ASR) are induced. The possible relationship of myelinic bodies, and other induced changes in cell wall ultrastructure, to PRs is worth further examination, especially in view of the extracellular location of the PRs. However, at this stage it remains completely possible that the myelinic bodies bear no relationship to either PRs or ASR.

Conclusion

The results discussed in this paper raise doubts about whether PRs are directly involved in ASR, and whether ASR should be regarded as a resistance mechanism at all.

Reduction in lesion number seems to be at least partly a result of altered leaf susceptibility to mechanical inoculation. This by some definitions is enhanced resistance. But as it is associated with decreased turgidity and raised levels of the stress hormone ABA, its disadvantages are likely to outweigh any marginal benefits in crop protection.

The evidence that PRs are involved in any specific way in limiting virus or lesion spread is not compelling. It is possible that ASR may limit lesion spread by general means such as altered cell wall properties or phenylpropanoid metabolism, without constituting a specific resistance mechanism. It is worth stressing that plants which develop ASR have already a constitutive and highly effective mechanism for preventing virus spread. ASR may simply represent a non-specific modulation of the expression of the primary mechanism. At this stage the question of whether PRs are an integral part of the localization of the initial infection becomes particularly relevant.

It is notable that treatments inducing ASR and PRs can cause some degree of stress. For example, growth of uninoculated leaves of 'White Burley' tobacco is inhibited when ASR is induced in them by lesion formation on lower leaves (Whenham and Fraser, 1981). Chemical treatments may cause some phytotoxic effects (e.g. Van Loon and Antoniw, 1982). These effects may prevent exploitation of the putative enhancement of resistance by ASR in crop protection.

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