

Pathogenesis-related proteins

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Occurrence and properties of pathogenesis-related proteins

In several plant species, upon infection with viruses, viroids, fungi or bacteria the development of symptoms is accompanied by the appearance of one or more new proteins. Such proteins were first identified in tobacco (*Nicotiana tabacum*) cultivars reacting hypersensitively to tobacco mosaic virus (TMV) (24, 50) and have now been detected in 16 plant species (Table 1), notably associated with necrotic reactions (35). The occurrence of these new proteins is not pathogen-specific, but determined by the type of reaction of the host plant, indicating that these proteins are of host origin. Since the proteins are induced under specific pathological conditions, they have been named pathogenesis-related proteins or PRs. All have been identified as additional bands present in polyacrylamide gels run either in the absence or in the presence of SDS and stained with general protein stains. Where further characterization of these proteins has been attempted, several common distinguishing properties have become evident, as exemplified by the PRs from tobacco (42, 44, 47) and bean (36).

1. Both tobacco and bean PRs elute later from gel filtration columns than the bulk of the protein from healthy leaves (5, 36, 42). This indicates that they must be relatively low-molecular-weight proteins and that their large separation upon electrophoresis in native polyacrylamide gels will be due mainly to charge differences. For the tobacco PRs this last conclusion has been borne out by the use of chromatofocusing, a column chromatographic technique in which proteins are separated according to their isoelectric points, which yielded pIs ranging

from 7.5 to 4.0 (33). A rather similar PR from tomato (p14) has a pI of 10.7 (10).

2. PRs are selectively extractable at low pH, where they remain soluble whereas the majority of the leaf proteins is not (25, 36, 44). This property considerably aids in their detection: whereas only four PRs are revealed upon electrophoresis of a total soluble protein extract from infected tobacco leaves, ten PRs can be identified as prominent bands when crude low-pH extracts are subjected to the same electrophoretic procedure.

3. PRs are highly resistant to the endogenous plant proteases as well as to commercial protease preparations including trypsin, chymotrypsin, papain, pronase, and proteinase K (33, 47). They are not protease inhibitors (34), nor are they produced by treatment of protein extracts from non-infected leaves with proteases.

4. At least in tobacco and cowpea (52), PRs are localized predominantly outside the cytoplasm in the intercellular spaces. They must thus be secreted through the plasmalemma, but, whereas three of the slow-moving PRs in tobacco may contain carbohydrate (33), at least the four tobacco PRs first identified are not glycoproteins, nor are they oxidases or hydrolases such as peroxidase, ribonuclease, protease, or phosphatase (42), which are known to be present in intercellular spaces. Their resistance to proteases is an obvious requirement for a function in this environment in which proteolytic enzymes abound.

Upon electrophoretic comparison, PRs from different plant genera display a similar but not identical range of relative mobilities in both native and SDS gels. Antisera against tobacco PRs did not react with PR preparations from other plant genera

(51). However, antiserum against tomato p14 reacted with several PRs from tobacco as well as one from cowpea (Nassuth & Sanger, personal communication), suggesting structural and possibly functional conservation among different plant genera.

Their characteristic properties together have allowed for an easy purification of some of the PRs from TMV-infected tobacco, starting from either low-pH extracts or intercellular fluid obtained through infiltration and centrifugation of leaf strips. By combinations of gel filtration and ion-exchange chromatography, four of the tobacco PRs have been purified (4, 5). Of these, PR-1a, -1b and -1c have similar molecular weights of about 14 200 (5) and their amino acid compositions are remarkably similar (4). PR-1a from 'Xanthi-nc' is typical with a high proportion of potentially acidic (29%) and aromatic residues (12%) but comparatively few basic residues (10%). The amino acid compositions are not identical, however, and even the corresponding PRs from different tobacco cultivars exhibit differences indicative of some amino acid substitutions. An antiserum raised against PR-1a from the cultivar 'Xanthi-nc' likewise reacted with PR-1a from the cultivar 'Samsun NN', and to a lesser extent with PR-1b and -1c, providing further proof that the members of the PR-1 group are closely related but not identical proteins.

Since reciprocal crosses between different *Nicotiana* species or tobacco varieties always give hybrids exhibiting the exact sum of the PRs of their two parents (3, 22), the present data support the concept that each PR is a product of a separate, though closely related gene.

Induction of pathogenesis-related proteins

Although PRs are particularly obvious when pathogens induce hypersensitive or systemic necrosis, necrosis or wounding is not a prerequisite for their appearance. However, because in tobacco the more slowly migrating PRs cannot always be easily resolved from proteins occurring in healthy plants, mostly only three or four PRs have been described to appear. At least PRs -1a, -1b, -1c and -2 appear spontaneously in healthy plants when these start to flower (17). PRs are also expressed in callus, whether this is habituated or grown on a hormone-

Table 1. Characteristics of pathogenesis-related proteins in different plant species.

Plant species	Number of PRs detected	References
<i>Apium graveolens</i>	1	(8)
<i>Citrus medica</i>	2-3	(8, 13)
<i>Cucumis sativus</i>	2-3	(8, 20, 41, 52, 53)
<i>Gomphrena globosa</i>	5	(37)
<i>Gynura aurantiaca</i>	2-3	(8, 13)
<i>Lycopersicon esculentum</i>	2-4	(8, 13, 16)
<i>Nicotiana debneyi</i>	1	(2, 3)
<i>Nicotiana glutinosa</i>	1	(2, 3, 50)
<i>Nicotiana rustica</i>	1	(41)
<i>Nicotiana sylvestris</i>	3	(3)
<i>Nicotiana tabacum</i>	9-11	(3, 24, 26, 46, 50)
<i>Nicotiana tomentosiformis</i>	1	(3)
<i>Phaseolus vulgaris</i>	4	(1, 36)
<i>Solanum demissum</i>	1	(8)
<i>Solanum tuberosum</i>	2	(13)
<i>Vigna sinensis</i>	2	(14)

containing nutrient medium (6, 7). In *Gomphrena globosa*, *Gynura aurantiaca* and tomato PRs may be produced during natural leaf senescence (9, 13, 31), and in cowpea and cucumber, PRs are induced by plasmolysis (52). Since PRs may also be induced artificially by the application of a variety of chemicals, notably polyacrylic acid (23), benzoic acid derivatives (54), high concentrations of various plant hormones (6, 45, 48), as well as culture filtrates from a pathogenic fungus (30) or bacterium (28), it has been proposed that induction might be the result of some type of stress (34, 53). Because ethylene is produced by most plant tissues as a general reaction to stress (55), and PRs can be induced in all tobacco's tested to date by ethephon, from which ethylene is released in the plant, as well as by the immediate natural precursor of ethylene: 1-aminocyclopropane-1-carboxylic acid (ACC), this gave rise to the idea that the induction of PRs is mediated by ethylene, at least in tobacco. Indeed, induction under all conditions investigated so far is associated with stimulated ethylene production, except for treatments with benzoic acid and its derivatives salicylic acid, aspirin, and 2,6-dihydroxybenzoic acid (48). When leaf discs infected with TMV were floated on a solution of aminoethoxyvinylglycine, which specifically blocks the formation of ACC in the biosynthesis of ethylene, the quantities of PRs induced due to virus infection

were substantially reduced. This observation clearly suggests that after virus infection, and most probably also when ethylene production is stimulated by chemicals, ethylene is the physiological inducer of PRs. The induction of PRs by benzoic acid, and particularly by its more effective derivative salicylic acid must then be explained by assuming that stimulated ethylene production triggers the synthesis of a, presumably aromatic, compound that mimicks the action of salicylic acid (49). Ethylene is known to stimulate aromatic biosynthesis in several plant species, including tobacco, and during the hypersensitive reaction of tobacco to TMV extensive changes in phenol metabolism have been observed (29).

Induction of PRs by ethephon or salicylic acid in tobacco is inhibited by cycloheximide or D-2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide, both inhibitors of protein synthesis on cytoplasmic ribosomes, but not by chloramphenicol, which inhibits protein synthesis in chloroplasts and mitochondria. Thus, cytoplasmic protein synthesis is necessary for PR appearance. Upon exposure of TMV-inoculated tobacco leaves to $^{14}\text{CO}_2$, at least PRs -1a, -1b, -1c and -2, as well as the most slowly migrating PR become radioactively labeled (33). When leaves were exposed to $^{14}\text{CO}_2$ at different times relative to the inoculation, the specific radioactivity of PR-1a altered comparatively little with changes in the time at which $^{14}\text{CO}_2$ had been administered, suggesting that, as necrotic lesions develop over a few days, the ^{14}C assimilated by the leaves was similarly available for PR synthesis, and that little, if any, turnover of PRs occurs. In this respect, PRs clearly differ from heat-shock proteins, which are rapidly synthesized upon a raise in temperature but subsequently disappear when adaptation has occurred (40).

When from infected tobacco leaves, poly A-mRNA was isolated and translated in cell-free protein-synthesizing systems, products migrating in polyacrylamide gels at the positions of PR-1a, -1b, -1c and -2 were precipitated with an antiserum against PR-1a (12), providing further proof both that PRs are synthesized *de novo*, and that these proteins are related. Recent evidence further indicates that PR-1a, -1b, and -1c are each translated from a separate mRNA (11).

In contrast to the effect of inhibitors of protein synthesis, inhibitors of DNA-dependent RNA-syn-

thesis, such as actinomycin D or cordycepin, do not inhibit the induction of PRs. Thus, for the expression of PRs no transcription is required. In agreement with this finding, Carr *et al.* (12) demonstrated that when poly A-mRNA from healthy leaves was translated *in vitro*, as much PRs were synthesized as from mRNA from infected leaves. These observations indicate that PR-mRNA is constitutively present, but not translated in non-stimulated leaves. Thus, the expression of PRs is controlled at the level of translation. A substantial increase in ethylene production apparently initiates a series of events which make the mRNAs available for translation.

Possible functions of pathogenesis-related proteins

If one assumes that PRs are synthesized in response to some type of stress, their function, analogous to the one postulated for heat-shock proteins (40), might be to protect the plant from extensive damage. During a hypersensitive reaction, PRs are first detectable in a ring around the necrosing center. In the subsequent phase of slow lesion expansion, they reach the highest concentration at the lesion margin. However, they also accumulate between lesions in the inoculated leaves and may appear in lower concentration in distant, non-infected leaves (38, 50). When non-infected tobacco leaves from plants previously inoculated with TMV and exhibiting a hypersensitive reaction, are challenge inoculated with TMV, smaller and occasionally fewer lesions develop than on similar leaves from plants previously treated with water (39). The association of the accumulation of PRs with the limitation of virus spread in the inoculated leaves, and of the appearance of PRs with the increased capacity to localize the virus in non-inoculated leaves, leads one to consider a role of PRs in limiting multiplication and/or spread of an invading pathogen. PRs would thus be responsible for the so-called 'systemic acquired resistance' (39), the phenomenon that due to a previous infection, plants become more resistant to further pathogenic attacks.

This hypothesis is supported by numerous findings that the presence of PRs and the state of acquired resistance are closely linked (21, 27, 43). Like the induction of PRs, acquired resistance is non-specific as to both the inducing and the challenging

pathogen, localized infection by one pathogen (virus, fungus, or bacterium) usually affording at least partial protection against both the same and other pathogens that induce similar symptoms. Chemicals that induce PRs also induce acquired resistance. When inducing chemicals are applied to leaves of the test plant, mostly PRs are produced only locally, apparently because these compounds are not readily transported symplastically to other plant parts. In plants thus treated, upon challenge, inoculation acquired resistance is likewise expressed only locally. Particularly striking is the close association between PRs and resistance in interspecific hybrids of *N. glutinosa* and *N. debneyi*. Upon infection with a necrotizing virus, both parent species produce the same single PR, but the hybrids contain this PR constitutively. When such hybrid plants are inoculated with TMV or tobacco necrosis virus (TNV), they develop very few, small local lesions from which little infectivity can be recovered (2). These hybrids thus possess both PRs and acquired resistance naturally without having been infected.

When *N. glutinosa* $\times$ *N. debneyi* amphidiploids are used as rootstock for grafting different other *Nicotianae*, the scions also start to express acquired resistance and PRs (22). These PRs are those characteristic of the scion. Hence, not the PRs themselves are transported, but a 'mobile compound' of unknown nature appears to move from the rootstock into the scion, transmitting the signal for the induction of PRs and enhanced resistance. Whereas in the hybrid this 'mobile compound' is present constitutively, in the parent species and in other *Nicotianae* it would only be formed upon appropriate stimulation. Presumably, the 'mobile compound' is not formed in sufficient amounts when PRs are induced chemically, since under those conditions PRs and resistance are largely confined to the treated areas. Only ethylene is exceptional in that local treatment with ethylene or ethephon induces both PRs and resistance in other leaves (49). Moreover, systemic acquired resistance induced by ethephon is stronger in upper than in lower leaves and both qualitatively and quantitatively similar to the systemic resistance induced by virus. Thus, the 'mobile compound' may be triggered by ethylene and function as an intermediary in its action of inducing PRs. The amphidiploid of *N. glutinosa* and *N. debneyi* does not produce elevated levels of

ethylene and it must be concluded that in these plants induction by ethylene is no longer required.

Although these observations strengthen the hypothesis that PRs are involved in acquired resistance, several findings are difficult to reconcile with this view (19). Whereas the induction of PRs in tobacco by salicylic acid is confined to the treated leaves, occasionally resistance is induced also in untreated upper leaves (49). This finding shows that the connection between the presence of PRs and the reduction in virus multiplication and spread may not be direct, and suggests that PRs are not responsible for acquired resistance. Similarly, after inoculation of tobacco with TMV, both in uninoculated halves of infected leaves and in upper leaves resistance became evident before PRs accumulated to any detectable level. Moreover, there was no correlation between the extent of resistance and the amount of PRs at the various times after primary inoculation. Conversely, plants treated with relatively low doses of methyl benzimidazol-2-yl-carbamate accumulated PR-1a, but resistance was not enhanced. In 'White Burley' tobacco, which does not localize TMV but forms necrotic lesions in response to TNV, PRs accumulated to similar extents as in TMV-localizing tobacco's. However, whereas the latter showed substantial systemic acquired resistance to TNV, in 'White Burley' resistance was not enhanced (32). Infiltration of tobacco leaves with a bacterial lipopolysaccharide fraction (LPS) results in the acquisition of systemic resistance to subsequent infection with *Pseudomonas solanacearum*. Although PRs were induced in tissues infiltrated with LPS, their presence was not correlated with the induced resistance to bacterial multiplication (28). Similar discrepancies have also been noted in *N. glutinosa* (18), cucumber (15, 20) and cowpea (15).

The *N* gene governing the necrotic lesion type of response to TMV, is temperature-sensitive in the sense that it confers hypersensitivity only at temperatures below 28 °C. At 30 °C no hypersensitive reaction occurs and the response of the plant is similar to that of tobacco varieties lacking the *N* gene: systemic mosaic symptoms develop and PRs are not induced. When PRs are induced by infection at 20 °C, their presence is not sufficient to prevent systemic spread of the virus upon a temperature shift to 30 °C. Thus, additional factors are necessary to determine virus localization. The oc-

currence of PRs merely reflects a particular type of stress which changes the metabolism of the plant and induces, on the one hand, PRs and, on the other hand, resistance to further infection.

Nevertheless, it is still possible that PRs may contribute to reducing the rate of spread of an invading pathogen. After induction in tobacco, PRs keep accumulating for several days without an increase in the extent of resistance expressed upon challenge inoculation. By far the largest part of the PRs is present in the intercellular spaces and it is difficult to envisage how those could be effective in reducing multiplication and spread of viruses present within the cells. Perhaps only the tiny fraction inside the protoplast is active. This would also explain why no relationship exists between the amount of PRs determined and the extent of resistance expressed. Moreover, instead of the well-characterized PRs one or more of the less readily detectable, more slowly migrating components might be the active ones. Further purification and characterization of the PRs as well as elucidation of their relationships and mode of synthesis and transport are now required to further probe their function in diseased and stressed plants.

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