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The induction of pathogenesis-related proteins by pathogens and specific chemicals

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Abstract

Pathogenesis-related proteins (PRs) are induced in tobacco and other plant species by both biotic and abiotic agents, comprising necrotizing and non-necrotizing viruses, viroids, fungi, bacteria, specific physiological conditions and a variety of chemicals. Both ethephon and the natural precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid, are good inducers and induction under all conditions investigated so far appears to be mediated by ethylene, except treatment with benzoic acid or its derivatives salicylic acid, aspirin, and 2,6-dihydroxybenzoic acid. Whereas the production of ethylene appears to result from a general reaction to stress, the mechanism by which *o*-hydroxylated benzoic acids induce PRs is different. In 'Samsun NN' tobacco, tobacco mosaic virus (TMV) and ethephon induce PRs in both the treated and the untreated leaves at 20 °C, but not at 32 °C. However, salicylic acid induces PRs only in the treated leaves, but is as effective at 32 °C as it is at 20 °C. It has been proposed, therefore, that ethylene leads to the temperature-sensitive synthesis of a, presumably aromatic, compound that mimics the action of salicylic acid and functions as the natural inducer of PRs.

The induction of PR 1a and 1b by salicylic acid or ethephon is blocked by cycloheximide but not by actinomycin D, whereas their accumulation upon TMV infection is inhibited up to 50% by actinomycin D. Actinomycin D similarly inhibits ethylene production in TMV-infected tobacco, supporting a role of ethylene in the induction of PRs in tobacco and indicating that ethylene acts by regulating the translation of the PR-mRNAs constitutively present but not translated in non-stimulated plants.

Additional keywords: tobacco, virus infection, ethylene, salicylic acid, stress, cycloheximide, actinomycin D.

Introduction

Since pathogenesis-related proteins (PRs) were first identified in hypersensitively reaction tobacco (Van Loon and Van Kampen, 1970; Gianinazzi et al., 1970), similar proteins have been found in several plant species after infection with different viruses, viroids, fungi, or bacteria (Van Loon, 1983). These proteins are characteristic of the host and are of host origin. Their occurence under different pathological conditions raises questions as to the factor responsible for their induction, and whether induction is always mediated by the same factor, or can be achieved independently by more than a single mechanism. Investigations pertaining to these questions have mostly been carried out with tobacco. This review will, therefore, be limited mainly to the induction of PRs in tobacco and refer only occasionally to other plant species.

Induction of PRs mediated by ethylene

Because upon gel electrophoresis of tobacco leaf proteins the more slowly migrating PRs are not always easily resolved from proteins present in healthy plants, usually only three or four PRs have been described. Several different conditions are now known to give rise to these PRs in tobacco (Table 1). Induction of PRs has been reported after infection with a number of different viruses inducing symptoms varying from a very faint green mottling, such as potato viruses X or Y, to systemic necrosis, such as produced by tobacco rattle virus. Although the proteins are induced in largest amounts when hypersensitive or systemic necrosis occurs, they are induced by non-necrotizing viruses in relatively small amounts at late stages of infection, notably when bright yellow symptoms occur. The occurrence of PRs in the absence of necrosis indicates that the appearance of these proteins is not simply a consequence of wounding or cell death. Although in hypersensitively reacting tobacco leaves the concentration of the proteins decreases rapidly with increasing distance from the lesions (Rohloff and Lerch, 1977), PRs are not confined to the inoculated leaves in which the virus is localized, but also appear in the rest of the plant, which remains symptomless. Hence, necrosis is not a prerequisite for PR induction, but when necrosis occurs, production of PRs appears to be stimulated.

Fungi and bacteria which cause a hypersensitive reaction also induce PRs. PRs are likewise induced when an insoluble fraction from *Nocardia asteroides* is injected into tobacco leaves, although this fraction does not induce any symptoms. Furthermore, PRs have been found in healthy plants when these are in the phase of monocarpic senescence, when they flower abundantly and then die. However, PRs do not occur in senescing leaves in the phase of sequential leaf senescence during the vegetative state, the presence of flowers being essential for induction (Fraser, 1981). Neither do PRs appear as a result of artificial ageing due to leaf detachment, mechanical or chemical wounding, or water, drought, or salt stress. This contrasts with the situation in *Gynura aurantiaca* (Conejero et al., 1979), *Gomphrena globosa* (Pennazio, 1981), and tomato (Camacho Henriquez and Sänger, 1982), where PRs may be produced during natural leaf senescence or ageing.

PRs are also found in healthy tobacco callus, whether this is habituated or grown on a hormone-containing nutrient medium. The occurrence of PRs in callus has been attributed to the presence of the cytokinin benzyladenine and the auxins 2,4-dichlorophenoxyacetic acid and indoleacetic acid in the medium, because these growth regulators by themselves, when injected in high concentrations into leaves, induce PRs. (Habituated callus produces its own auxins and cytokinins). Like benzyladenine, the cytokinin kinetin also induces PRs in tobacco.

In addition, several other chemicals induce PRs when injected into or sprayed onto leaves, watered on the soil, or taken up by detached leaves through the petiole or by leaf discs floating on the solution. Polyacrylic acid is effective only in the tobacco cultivars Xanthi and Xanthi-nc, and although it may act as an inducer in some other plant species, its effect is not a general one. In contrast, PRs are induced in all tobacco's tested to date by ethephon, from which ethylene is released in plant tissues, as well as by the immediate natural precursor of ethylene: 1-aminocyclopropane-1-carboxylic acid (ACC). Similarly active are benzoic acid and its more potent derivatives salicylic acid, aspirin, and 2,6-dihydroxybenzoic acid. In *Gomphrena globosa,* however, aspirin

Table 1. Induction of pathogenesis-related proteins and stimulation of ethylene production in tobacco.

Inducer of PRs	Reference	Ethylene production[a]
Necrotizing viruses		
Tobacco mosaic virus	Van Loon and Van Kammen, 1970	+
Tobacco necrosis virus	Van Loon, 1975	+
Tobacco rattle virus	Van Loon, 1975	n.d.
Potato virus Y^n	Van Loon, 1975	n.d.
Tomato bushy stunt virus	Pennazio et al., 1983	n.d.
Non-necrotizing viruses		
Potato aucuba mosaic virus	Kassanis et al., 1974	n.d.
Potato virus X	Kassanis et al., 1974	n.d.
Potato virus Y	Kassanis et al., 1974	n.d.
Cucumber mosaic virus	Kassanis et al., 1974	n.d.
Alfalfa mosaic virus	Kassanis et al., 1974	n.d.
Fungi		
Thielaviopsis basicola	Gianinazzi et al., 1980	n.d.
Bacteria		
Nocardia asteroides extract	Gianinazzi and Martin, 1975	n.d.
Pseudomonas syringae	Ahl et al., 1981	n.d.
Physiological conditions		
Flowering	Fraser, 1981	n.d.
Callus	Antoniw et al., 1981	+
Chemicals		
Polyacrylic acid	Gianinazzi and Kassanis, 1974	n.d.
Ethephon	Van Loon, 1977	+
1-aminocyclopropane-1-carboxylic acid	Van Loon, unpublished	+
Acetylsalicylic acid	White, 1979	n.d.
Salicylic acid	White, 1979	0
Benzoic acid	White, 1979	0
2,6-Dihydroxybenzoic acid	Van Loon, unpublished	0
Benzyladenine	Antoniw et al., 1981	+
Kinetin	Van Loon, unpublished	+
Indoleacetic acid	Antoniw et al., 1981	+
2,4-Dichlorophenoxyacetic acid	Antoniw et al., 1981	+
Barium salts	White and Antoniw, 1981	+
Cobalt salts	White and Antoniw, 1981	−[b]
Manganese salts	White and Antoniw, 1981	+
Mannitol	Pierpoint et al., 1981	n.d.
Methyl benzimidazol-2yl-carbamate	Fraser, 1982	n.d.
Phytic acid	Van Loon, unpublished	+

[a] +: Increase; 0: No change; −: Decrease; n.d.: Not determined.
[b] Under conditions where no PRs were induced.

was found to be inactive (Pennazio and Redolfi, 1980). PRs have further been induced by barium, cobalt and manganese salts, whereas silver salts were reported to induce PRs in *Gynura aurantiaca* (Conejero et al., 1980). Mannitol, when applied at hypertonic concentrations, was found to be an effective inducer in both cowpea and cucumber (Wagih and Coutts, 1981), but induces PRs only occasionally and in small amounts in tobacco. Furthermore, methyl benzimidazol-2yl-carbamate (MBC), which has cytokinin-like properties, and phytic acid are relatively good inducers when applied in concentrations up to 1 mM.

Although potato virus X was reported by Kassanis et al. (1974) to induce PRs, Van Loon (1975) was unable to confirm this observation. This discrepancy may be due to the use of different strains of the virus. Similarly, L.C. van Loon (unpublished results) was unable to induce PRs by floating leaf discs on 10 mM $CoCl_2$, whereas White and Antoniw (1981) obtained induction by injection of this solution into leaves. Thus, not only are not all inducers active in all plant species, but also, within a single species, inducers are not active under all conditions. Moreover, in tobacco the different inducers do not induce all PRs in the same relative proportions, and these proportions also vary depending on the physiological state of the plant tissue. The significance of these differences is not clear, but from the reproducible characteristics of the gel electrophoretic patterns it appears that the accumulation of PRs is strictly controlled and that patterns exist according to which induction of PRs is regulated.

When tobacco leaf discs were floated on kinetin solutions in concentrations ranging from 10 to 200 μM, at least PR-1a and -1b accumulated in increasing amounts with increasing kinetin concentration (Fig. 1). Over this range, kinetin also induced the production of increasing amounts of ethylene (Fig. 2A). Similarly, the other cytokinins and the auxins (cf. Fig. 2B) all induced the production of large amounts of ethylene. Large quantities of ethylene are likewise produced naturally by tobacco callus cultures. Since both ethephon and ACC are good inducers of PRs, the induction of PRs by auxins and cytokinins appears to be mediated by ethylene. That ethylene may also be the physiological inducer of PRs in hypersensitively reacting plants can be inferred from the following observations. The appearance of local lesions in hypersensitively reacting tobacco is accompanied by a large burst of ethylene, after which PRs start to appear (Pritchard and Ross, 1975; Van Loon and Van Kammen, 1970). The formation of the ethylene precursor ACC can be specifically blocked by aminoethoxyvinylglycine (AVG). When leaf discs infected with TMV were incubated on AVG, the quantities of PRs induced due to virus infection were considerably reduced. Also in other experiments, generally a good quantitative relationship exists between the amount of ethylene evolved and the quantities of PRs induced. Since ethylene is produced upon infections with viruses causing chlorosis (Bailiss yet al., 1977) or necrosis (De Laat and Van Loon, 1983), these results clearly suggest that after virus infection ethylene is the physiological inducer of PRs.

Although mannitol has been reported to stimulate ethylene production in *Citrus* leaf discs (Riov and Yang, 1982), it has not yet been investigated whether it also stimulates ethylene production in tobacco. Since neither salt stressing intact plants, nor floating leaf discs on salt solutions (up to 0.7 M) induced PRs, osmotic effects do not seem to be involved. Rather, induction might be the result of some type of stress (Pierpoint et al., 1982; Wagih and Coutts, 1982). It may be significant that ethylene is produced by most tissues as a general reaction to stress (Yang and Pratt, 1978). Stress

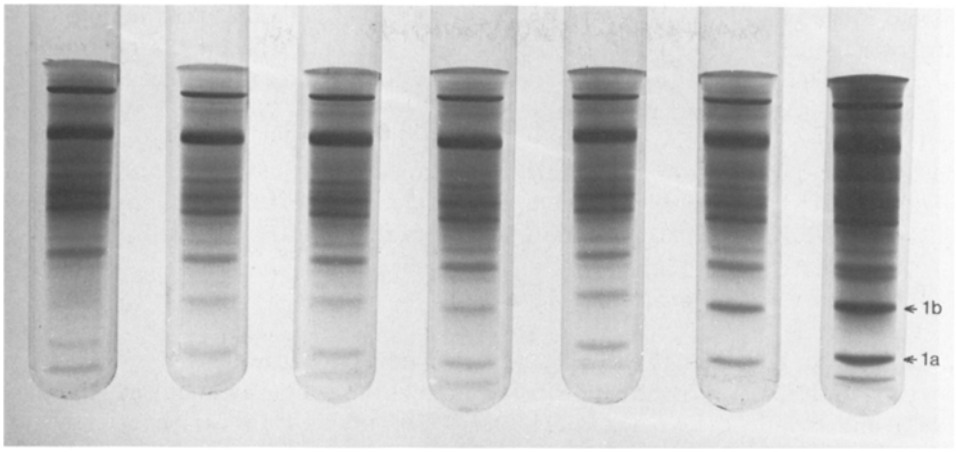


Fig. 1. Electrophoretic patterns in 10% polyacrylamide gels of pH 3-soluble proteins from 'Samsun NN' tobacco leaf discs floated for 7 days on (from left to right) water (control), 10, 20, 50, 100 and 200 μM kinetin, and water but inoculated with TMV. The proteins in the lower part of the gel from the control are present in small amounts in healthy leaves; they are not PRs.

has been invoked to explain the induction of PRs by MBC (Fraser, 1982) and also appears to be the cause of the induction by the metal salts. These salts proved highly toxic to floating leaf discs and on $BaCl_2$ and $MnCl_2$ large amounts of ethylene were produc-

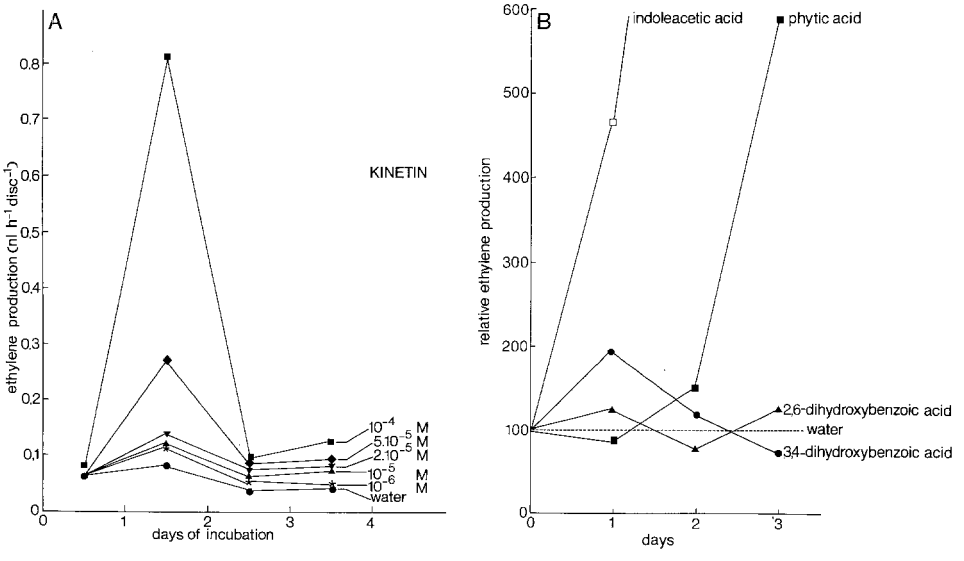


Fig. 2. Ethylene production of 'Samsun NN' tobacco leaf discs floated on (A) water (●) or different concentrations of kinetin, and (B) 100 μM indoleacetic acid or 1 mM phytic acid, 3,4-dihydroxybenzoic acid or 2,6-dihydroxybenzoic acid, relative to water (100%).

ed and PRs accumulated. However, on $CoCl_2$ neither stimulation of ethylene production, nor induction of PRs occurred. Since Co^{2+} is an effective inhibitor of the last step of ethylene synthesis, these observations support the idea that ethylene mediates the effect of stress in the induction of PRs.

The differential induction of PRs in different tobacco cultivars by polyacrylic acid needs further investigation, particularly in relation to a possible differential induction of ethylene. Large amounts of ethylene were produced by leaf discs floating on 1 mM phytic acid (Fig. 2B), at which concentration PRs were readily induced.

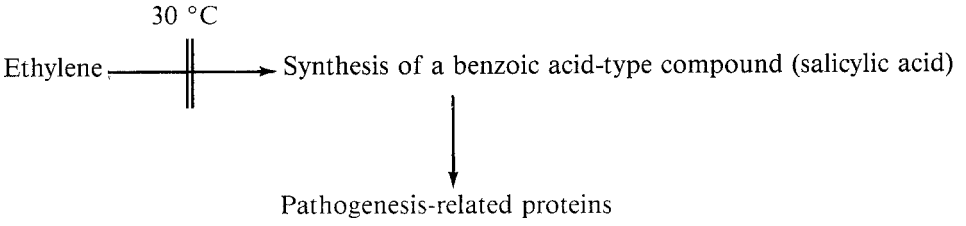
Induction of PRs by benzoic acid derivatives

In contrast to the many cases of treatments inducing both ethylene and PRs, benzoic acid and its derivatives 2-hydroxybenzoic acid (salicylic acid) (Van Loon and Antoniw, 1982) and 2,6-dihydroxybenzoic acid (Fig. 2B) induced PRs without inducing ethylene. Induction of PRs was restricted to derivatives containing a hydroxyl group exclusively at the ortho-position, 2,3-, 2,4- and 2,5-dihydroxybenzoic acids being virtually inactive. Like 3,4-dihydroxybenzoic acid, these latter derivatives are oxidized by peroxidase present in the intercellular space, giving rise to reddish-brown products. Some stimulation of ethylene production may occur under these conditions (Fig. 2B) and then traces of PRs become apparent. However, the mechanism by which the *o*-hydroxylated derivatives induce PRs is clearly different in that the induction by ethylene is by-passed.

The induction of PRs by ethephon and salicylic acid was further compared at different temperatures in the tobacco cultivar Samsun NN, which by virtue of the presence of the gene *N* reacts hypersensitively to tobacco mosaic virus (TMV). The *N* gene is temperature-sensitive: at temperatures above 28 °C no hypersensitive response occurs and the reaction of the plant is similar to that of tobacco's lacking this gene. Systemic mosaic symptoms develop and PRs are not induced.

Treating tobacco leaves with either ethephon or salicylic acid at 20 °C induced PRs in the treated leaves up to the level induced by virus infection. Ethephon further mimicked TMV infection in inducing PRs in both the treated and the untreated leaves at 20 °C, but not at 32 °C. In contrast, salicylic acid induced PRs only in the treated leaves, but proved to be as effective at 32 °C as it was at 20 °C (Van Loon and Antoniw, 1982). These observations indicate that the induction of PRs itself is not a temperature-sensitive process, but that the induction by salicylic acid is limited to the area exposed. Presumably, salicylic acid, when injected into a leaf, is not taken up into the phloem and, therefore, remains restricted to the treated area.

The differing results obtained with ethephon and salicylic acid may be unified by adopting the following hypothetical scheme of events:



Ethylene, produced naturally during the hypersensitive reaction of tobacco to TMV, leads to the temperature-sensitive synthesis of a, presumably aromatic, compound, that mimics the action of salicylic acid and functions as the natural inducer of PRs. Stimulation of aromatic biosynthesis by ethylene has been reported in a number of cases, including tobacco (Reuveni and Cohen, 1978), but nothing is known about the intrinsic temperature sensitivity of this response.

Any condition leading to ethylene production above a certain threshold may thus induces PRs in tobacco. This conclusion may be further generalized, as induction of PRs by ethephon is not confined to tobacco. In 'Pinto' beans infected with necrotizing viruses such as tobacco necrosis virus, at least three or four proteins extracted at low pH are greatly stimulated, if not newly induced (Redolfi and Cantisani, 1981). Pricking bean leaves with ethephon caused identical qualitative changes in the protein pattern, although the relative proportions of the bands were greatly different. Even salicylic acid appeared to show some activity in that traces of the most rapidly migrating band became apparent after spraying of healthy plants.

Effects of inhibitors of RNA and protein synthesis on induction of PRs

Since inhibitors of protein synthesis block virus multiplication, their use in assessing whether PRs are synthesized de novo in virus-infected plants has been precluded. However, since the induction of PRs appears to be mediated by ethylene and both ethephon and salicylic acid are potent chemical inducers, this question has now become amenable to experimentation. When salicylic acid was used as the inducer by floating tobacco leaf discs on a 1 mM solution, PR 1a and 1b accumulated to substantial amounts between 2 and 5 days of incubation. This accumulation was reduced in a concentration-dependent manner when, together with salicylic acid, cycloheximide was applied in a concentration range up to 20 $\mu g.ml^{-1}$. Cycloheximide did not affect total protein content of the leaf discs for the duration of the experiment, and pH 3-soluble protein decreased more slowly than both PRs with increasing concentration of the drug. Whereas low doses of cycloheximide strongly inhibited the accumulation of PR 1a and 1b, the more slowly migrating PRs increased until 10 μg cycloheximide ml^{-1} but decreased rapidly at the higher concentrations of the drug. These observations are suggestive not only of de novo synthesis of PRs but also of a modulation of the amount of individual PRs when general protein synthesis is perturbed.

It has been reported (Van Loon and Van Kammen, 1970) that actinomycin D inhibits the accumulation of PRs in TMV-infected 'Samsun NN' tobacco. This inhibition was, however, rather small and never exceeded 50%. Similar results were also described by Kassanis and White (1974) for the induction of PRs by polyacrylic acid. However, the induction of PRs by salicylic acid or ethephon was not inhibited by actinomycin D, indicating that for the expression of PRs no transcription is required. These findings are in agreement with the conclusion of Carr et al. (1982) that mRNA coding for PRs is constitutively present but not translated in non-stimulated plants. Thus, the expression of PRs is controlled at the level of translation. In TMV-infected tobacco actinomycin D must, therefore, block a transcription-dependent step prior to the release of PR-mRNA from translation inhibition. Since actinomycin D did not inhibit the induction of PRs by ethephon, the transcription-dependent step must be at or before the level of ethylene. Indeed, actinomycin D inhibits ethylene production in TMV-

infected tobacco, but only to a limited extent (De Laat and Van Loon, 1983) similar to the extent of inhibition of PR production (Van Loon and Van Kammen, 1970). These results again support a role of ethylene in the induction of PRs in tobacco and indicate that ethylene acts by regulating the translation of PR-mRNA.

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