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Review article

Function and Regulated Accumulation of Plant Pathogenesis-Related Proteins

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Abstract

De novo synthesis of pathogenesis-related (PR) proteins occurs in a plant after pathogen infection. There are at least two general independent pathways of PR proteins induction, utilized by the plant. One pathway is responsive to ethylene and appears to be light-dependent, while the other pathway is both ethylene and light-independent. In addition to their induction, as a result of pathogenesis, the PR proteins were also found to accumulate in a developmental-dependent fashion, during flower development. The differential compartmentalized expression of the PR proteins in the floral tissues differ from their concerted appearance in pathogenesis. Their unique appearance in the reproductive organs, implies that they fulfill another function, which is not pathogen-related, but rather associated with reproductive physiology.

Keywords: ethylene, plant pathogenesis, xylanase, mycorrhizal

1. Introduction

Infection of hypersensitive cultivars of *Nicotiana tabacum* by the Tobacco Mosaic Virus (TMV), induces the accumulation of a group of proteins called pathogenesis related (PR) proteins (Parent and Asselin, 1983; Van Loon and Van Kammen, 1970; Van Loon et al., 1987). PR proteins have been associated with systemic acquired resistance and incipient antipathogen effects (Van

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Loon, 1982; Richardson et al., 1987). Related proteins have been identified in many plant species including monocot (Fink et al., 1988; Nasser et al., 1988) and dicot plants (Bol and Van Kan, 1988). Best characterized in tobacco, PR proteins fall into five major groups, which are coordinately regulated in response to infection by fungi, bacteria and viruses (Van Loon et al., 1987). The term PR proteins was originally restricted to a group of acidic proteins, which accumulated in the extracellular space of infected leaves and were easily resolved by native polyacrylamide gel electrophoresis. Recent evidence has shown that the acidic polypeptides are a subset of intermediate size gene families, which have both acidic and basic protein counterparts. The PR proteins accumulate in different intracellular locations. They display varied tissue specificities and show complex modes of pathogen induced and developmental regulation.

In this article we will focus on three PR protein groups, for which data on the gene as well as the gene product are available. One group, consisting of both acidic and basic PR proteins forms, has been shown to have (1–3)-β-glucanase activity (Kauffmann et al., 1987). The acidic polypeptides of this group are PR-2,N,O. Another group consisting of two acidic and two basic proteins has endochitinase activity. The acidic polypeptides of this group are PR-P and Q (Legrand et al., 1987). The third group, for which no enzymatic function has been defined, consists of at least three acidic isoforms PR-1a, b, c and an unknown number of basic isoforms. The enzymatic activities ascribed to some PR proteins clearly point to their role in the general defense responses of the plant. However, in the light of recent data revealing unique developmental and cellular specificities in PR proteins accumulation, the possibility exists that different subsets of these proteins serve other biological functions.

2. Induction Patterns of PR Proteins

There are two independent pathways for PR proteins elicitation

PR proteins have been detected in leaves infected by pathogenic bacteria, fungi or viruses. TMV infection of a hypersensitive cultivar of *Nicotiana tabacum* results in the concomitant accumulation of three groups of acidic PR proteins as shown in Fig. 1. Accumulation of the proteins can also be induced by a number of elicitors of biotic or chemical origin, notably salicylic acid, some amino acid derivatives (White, 1979; Asselin et al., 1985) and high concentrations of certain plant hormones (Memelink et al., 1987; Shinshi et al., 1987).

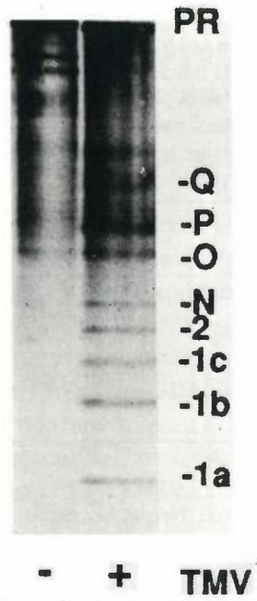

Figure 1. Coomassie blue staining of non-denaturing gel electrophoresis of extracellular proteins from TMV infected (+) and non-infected (−) leaves of *N. tabacum* NN. leaves were inoculated at 200 lesions/leaf and extracellular proteins were isolated and fractionated as described (Lotan et al., 1989).

Ethylene was suggested to be the natural mediator of the induction of the PR proteins. Exogenous application of the spontaneous ethylene producer, ethephon, induced the production of PR proteins (Van Loon and Antoniw, 1982). Ethylene treatment of tobacco plants markedly increased the glucanase content of the leaves (Felix and Meins, 1987). Application of aminoethoxyvinylglycine (AVG), which specifically blocks the formation of 1-aminocyclopropane-1-carboxylic acid (ACC), a substrate in the biosynthesis pathway of ethylene, have shown reduced amounts of PR proteins from viral infected plant tissue (Van Loon, 1985; Lotan and Fluhr, 1990). The inhibitory effect AVG has on induction of PR proteins is consistent with a physiological role for ethylene in the induction process. The appearance of PR proteins during senescence (Camacho-Henriquez and Sänger, 1984; Fraser, 1981) further supports such a role, since ethylene is known to be synthesized during that stage of plant maturity. However when two additional elicitors of PR accumulation, *Pseudomonas syringae* pv. *tabaci*, a pathogen of tobacco, and (1-4)-β-endoxylanase, an enzyme isolated from *Trichoderma viride* cultures were tested in the presence of AVG or silver thiosulfate (a compound which inhibits ethylene action), the

Table 1. Induction of PR proteins by biotic and chemical elicitors. Leaves were treated with either; TMV, 200 lesions/leaf; α-aminobutyric acid, 1 mM; xylanase, 1 ng per injection site or *P. syringae*, 10^{-5} OD per injection site. Leaves were harvested 24 hr later except in the case of TMV (48 hr later) and total leaf extracts were fractionated on denaturing polyacrylamide gels. Immunoblots were prepared and developed with the appropriate antisera.

Inducer	PR proteins			Ethylene-Dependent	Light-Dependent
	1-a,b,c	P,Q	2,N,O		
TMV	+	+	+	+	+
α-Aminobutyric acid	+	+	+	+	+
Xylanase	+	+	−	−	−
P. syringae	+	+	−+	−	−

accumulation of PR proteins was unaffected. Hence, regulatory pathways of PR proteins induction can be ethylene independent (Table 1).

For a large variety of chemical elicitors the presence of light and green photosynthetically competent tissue had been shown to be correlated with PR proteins accumulation (Asselin et al., 1985). We have recently shown that a photosynthesis deficient mutant (chlorophyll-less albino cytoplasmic mutant) could effectively synthesize PR proteins. In addition, elicitors of PR proteins synthesis, which belong to the class that are ethylene independent, were found to be active in the dark as well (Table 1; Lotan and Fluhr, 1990). A result which shows that it is the transduction pathway which exhibits light dependency rather than the synthesis of PR proteins themselves; collectively these experiments suggest the existence of two distinct pathways for inducing PR proteins. One pathway is ethylene-dependent and shows a light requirement. Its activation is correlated with viruses or chemicals which initiate a hypersensitive response. The other induction pathways initiated by xylanase and *P. syringae* is ethylene-independent and shows no light requirement. In pathogen-related responses the coordinate accumulation of at least three groups of the PR proteins, namely PR 1a, 1b, and 1c (which has unknown biological function), the (1-3)-β-glucanases group and the endochitinases group, is the general rule (Van Loon, 1985; Rigden and Coutts, 1988; Asselin et al., 1985; Parent and Asselin, 1983). However, *P. syringae* and endo (1-4)-β-xylanase were found to induce only two groups: PR-1 and the the endochitinases (Table 1; Lotan and Fluhr, 1990). Salicylic acid, a chemical elicitor, was suggested to induce only PR-1 mRNAs (Hooft van Huijsduijnen et al., 1986). These data suggest independent regulatory pathways for some PR groups and underlines the hitherto unexpected complexity in their regulation.

While we have restricted our discussion to PR proteins, it is important to keep in mind that plants react in a broad avenue of defense postures. It is of interest to see whether the plant utilizes the same transduction pathway for different defense mechanisms as well. We have found recently, in tobacco, the concomitant appearance of PR proteins together with the tobacco phytoalexin capsidiol (A. Sharon, in preparation). Capsidiol belongs to the sesquiterpenoid class of antibiotics. The induction kinetic of phytoalexin accumulation showed parallel control by ethylene-dependent and independent pathways in correlation with the type of elicitor used. It is not clear whether factors common in the elicitation of other pathways also function in PR proteins accumulation. Cell wall glucans, liberated from the pathogen as a result of the action of plant-derived hydrolases, or glucans liberated by the plant cell wall as a result of pathogen-based hydrolases, are thought to play an integral role in plant pathogen interaction (Ryan, 1987). Similar roles for complex glucans have not been reported in the induction of PR proteins. A glucan preparation from *Phytophthora megasperma* f. sp. *glycinea*, which is active in elicitation of phytoalexin synthesis in soybean, has recently been shown to induce resistance against virus in tobacco, when applied within a few hours of TMV inoculation (Kopp et al., 1989). However, it did not induce the accumulation of PR proteins in tobacco. Conceivably xylanase action in plant cell walls could release active polysaccharides. However, plausible substrate or enzymatic products which are xylan and xylose oligomers, respectively, were ineffective in PR proteins induction (Lotan, unpublished results).

Under some conditions PR proteins are expressed constitutively

While acidic PR proteins were originally identified by their accumulation in the extracellular fluids of elicitor induced leaves, recent findings have underscored other modes of expression in both the acidic and the basic PR proteins classes. High levels of basic RNA transcripts of chitinase and glucanase have been reported to occur constitutively in the roots (Memelink et al., 1987; Shinshi et al., 1988). Under certain conditions acidic type of PR proteins are constitutively expressed. In *N. glutinosa* and *N. debneyi* an immunoreactive polypeptide comigrates with PR-1 and is induced upon TMV inoculation (Fig. 2). However, an interspecific hybrid produced by Ahl and Gianinazzi (1982) shows constitutive expression (Fig. 2). The hybrid plant is naturally resistant to certain pathogen infections. The small size of the hybrid plants may indicate aberrant hormone balance, which would explain the enhanced basal level of PR accumulation. Its utility in plant breeding programs has not been shown.

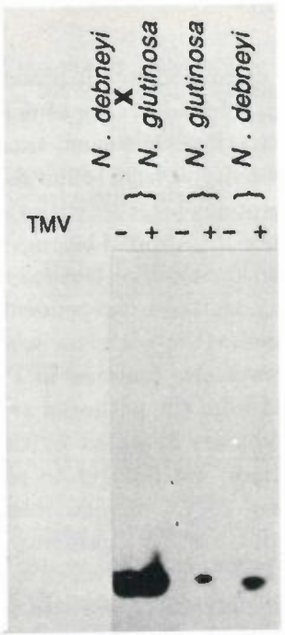

Figure 2. Constitutive and induced accumulation of PR-1 in two tobacco species, *N. glutinosa* and *N. debneyi* and their hybrid progeny. Immunoblots of denaturing polyacrylamide gels were prepared as described (Lotan et al., 1989) and reacted with antisera to PR-1. (−) are control plants, (+) are plants inoculated with TMV.

Developmental regulation of PR proteins

PR proteins are expressed during normal plant development. Low levels of PR-1a, b, c and PR-2 (glucanase) accumulate in leaves of healthy plants as they begin to flower and senescence (Fraser, 1981). RNA specific for the chitinases group was detected in healthy tobacco flowers (Memelink et al., 1987). Further analysis of fully developed tissues of healthy flowers revealed an unusual pattern of PR proteins accumulation (Lotan et al., 1989). PR-1 group appeared in sepal tissue, a glycosylated PR-2,N,O-type (glucanase) was found in pistillar parts, while PR-P,Q-type (chitinase) was present in pedicels, sepals, anthers and ovaries (Fig. 3). The developmental dependence of their appearance was exemplified with the help of a homeopathic cytoplasmic male sterility mutation present in *N. tabacum*. In these mutant plants the stamens are malformed and differentiate into atrophied stigmatoid filaments (Aviv and Galun, 1986). However, the PR proteins which accumulated in the latter pistillar structures were of PR-2,N,O type and not the PR-P,Q type seen in normal anthers (Lotan et al., 1989). Evidently, PR proteins accumulation in

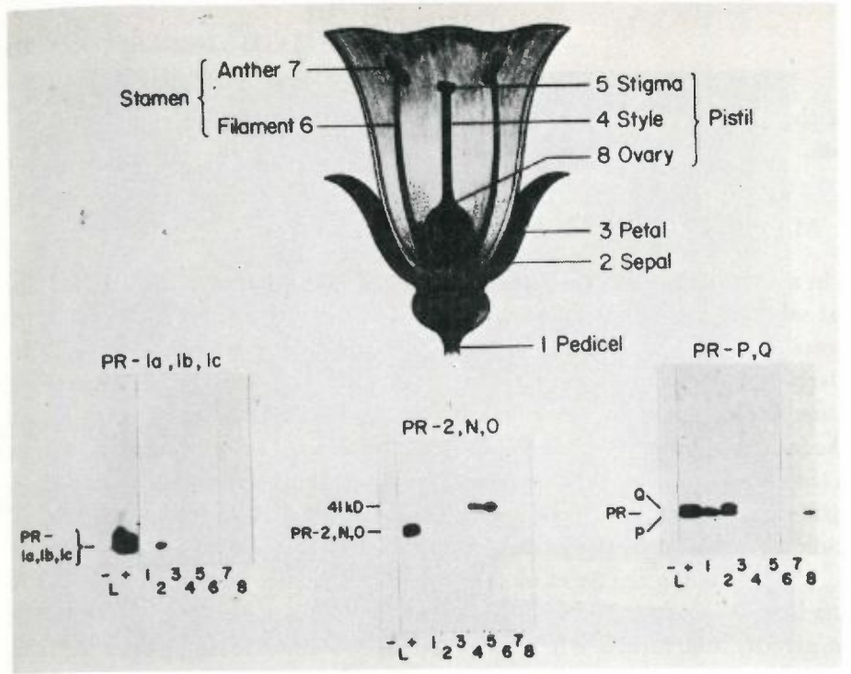

Figure 3. Flower-specific expression of PR proteins. The flower was divided into various tissues and protein extracts were analyzed for the presence of PR-1 group, PR-2,N,O or PR-P,Q. The data is taken from Lotan et al. (1989). L is leaf with (+) or without (−) inoculation of TMV.

the flower is determined by the final organ structure and not its ontogenic origin.

Although the expression of PR genes is under a developmental mode of regulation in flower organs, these organs are still capable of reacting to pathogen stress. When pathogenesis occurs the accumulation of PR proteins in flower parts is identical to those found in the leaves (Lotan et al., 1989). As the PR proteins belong to multi-gene families, one could ask whether the very same members of the gene family are being produced in each expression mode, i.e. pathogenesis versus developmental, or whether different members are expressed each time. This question has been addressed at the gene level in the PR-2,N,O class. We have recently established, using cDNA clones that the genes, which are expressed under pathogenic induction in the leaves and the genes appearing in the flowers, are highly homologous but not identical (N. Ori, in preparation). It appears that within this gene family, tissue specific accumulation, as compared to pathogen modification has been achieved by gene duplication and promoter modification. Whether this will prove to be the case

for the other acidic PR proteins families detected in the flower, remains to be seen.

3. Modes of PR Proteins Action

In a pathogen-based reaction acidic PR proteins are expressed in the whole leaf without any apparent cell specificity. Each cell is thought to release PR proteins to the extracellular space. PR-1 polypeptides accumulate extracellularly in isolated protoplasts (Carr et al., 1987) and are present in the conditioned medium of *N. tabacum* cell cultures (T. Lotan, unpublished results). The location of the basic polypeptide counterparts of PR proteins is not known. In the case of glucanase, it has recently been established that the acidic and the basic isoforms display different subcellular localization in tobacco plants. The acidic isoforms are located extracellularly (Kauffmann et al., 1987; Legrand et al., 1987; Van den Bulcke et al., 1989), while the basic isoform of glucanase occurs intracellularly in the vacuoles (Van den Bulcke et al., 1989). Interestingly, the glycosylated form of PR-2,N,O-type that accumulates in pistillar structures has been detected intracellularly (Lotan et al., 1989). The basic form of the chitinases has been localized to vacuolar structures in bean (Boller and Vogeli, 1984), but their localization in tobacco has not yet been established.

Possible anti-viral and anti-fungal effects of PR proteins

Plants infected by TMV or by other pathogens that elicit a hypersensitive response produce PR proteins in the leaves infected by pathogen, as well as in other non-infected leaves of the same plant (Parent and Asselin, 1983). The putative systemic messenger is unknown. Following the systemic appearance of these proteins, there is an increase in resistance of the plant to further infection by various unrelated pathogens (White, 1979; Ahl and Gianinazzi, 1982; Hooft van Huijsduijnen et al., 1986). Constitutive expression of PR proteins in a cross of *N. glutinosa* and *N. debneyi* resulted in heightened resistance to TMV (Gianinazzi and Ahl, 1983). However, no quantitative nor temporal relationship was found between PR proteins and resistance (Fraser, 1982; Van Loon, 1985). There is also no correlation between the appearance of the systemic necrosis after pathogenic infection and the accumulation of the PR proteins (Pennazio et al., 1983). RMB_7 is a cross between *N. rustica* and *N. tabacum* var. *Maryland Mammoth* (Smith, 1973; Martin-Tanguy, 1985). The hybrid is not hypersensitive to TMV inoculation as is shown in Fig. 4. Instead, a necrotic region continues to enlarge around the inoculated site. Nevertheless, PR-1 group, as indicated in Fig. 5, accumulates in the

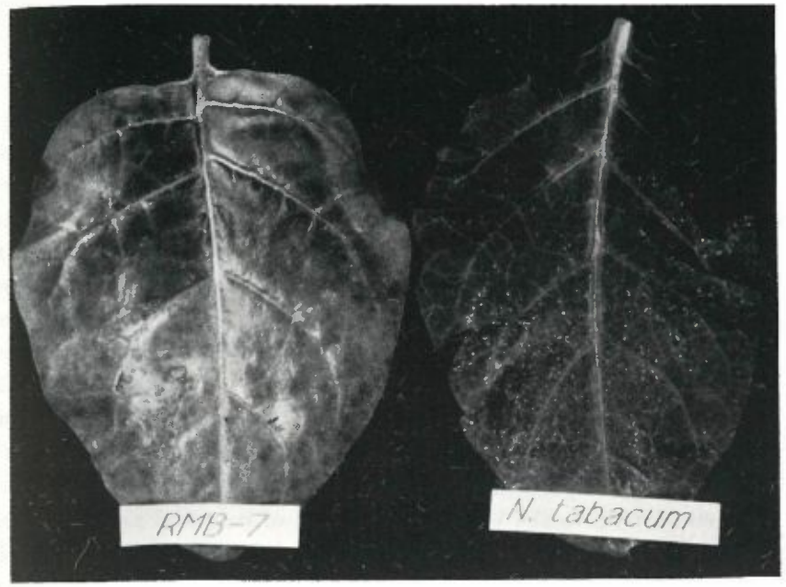

Figure 4. Inoculation of RMB_7 and *N. tabacum* with TMV. Local lesions are visible as white dots on the leaf from *N. tabacum*. The same inoculum on RMB_7 causes local lesions that continue to grow and spread.

Figure 5. Induced accumulation of PR-1 group in RMB_7 inoculated with TMV. Immunoblots of denaturing polyacrylamide gels were prepared as described (Lotan et al., 1989) and reacted with antisera to PR-1.

infected plant. Moreover, a transgenic plant expressing constitutively PR-1a is not resistant to TMV infection (Linthorst et al., 1989). Taken together, the physiological and genetic evidence do not support the conjecture that the presence of PR proteins, in themselves, is indicative of a plant's anti-viral state. The resistance to virus infection in induced tobacco may be the result of a combined effect of some of the PR proteins and additional defense systems of the plant. As mentioned in the previous section, the application of a glucan preparation from *Phytophthora megasperma* f.sp. *glycinea* has recently been shown to induce resistance against viruses in tobacco (Kopp et al., 1989). However, it did not induce the accumulation of PR proteins in tobacco. A situation in which viral resistance was found to be divorced from PR proteins accumulation was achieved by using the fungicide methyl benzimidazol-2yl-carbaamate (MBC) as the elicitor of resistance (Fraser, 1982).

The polysaccharide hydrolyzing enzymes, i.e. chitinases and the (1-3)-β-glucanases, could be involved in the degradation of bacterial and fungal cell walls or even insect exoskeletons. *In vitro* experiments have shown the inhibitory effect of chitinase on fungal growth. When chitinase was used in combination with glucanase, a broadened range of pathogen inhibition was obtained (Schlumbaum et al., 1986; Mauch et al., 1988).

Do PR proteins play a role in mycorrhizal colonization?

The establishment of symbiotic associations, such as those present in vesicular-arbuscular mycorrhizae, necessitates extensive colonization of root tissue. Spanu et al. (1989) undertook to study whether the invasion of a mutualistic symbiont, *Glomus versiforme* (Karst) Berch., induced PR proteins in the mycorrhizal *Allium porrum* L. They found that during initial penetration of the outer cortex the host roots accumulated chitinase. However, in later stages a decrease in enzyme activity was noted, despite the continued massive colonization of the roots by fungus and the continued differentiation of fungal arbuscules. The chitinase was detected immunohistochemically and appeared to be localized to the vacuoles. The cellular compartmentation of chitinase and lack of *in vitro* binding of chitinase by fungal hyphae led the researchers to conclude that it is unlikely that plant chitinase is involved in the development of the mycorrhizal fungus.

Do PR proteins play a role in the physiology of plant reproduction?

The temporal and spatial gene expression programs that characterize flower development has been documented in the last few years (Drews and Goldberg,

1989). Several stylar glycoproteins were recently shown to be associated with the phenomenon of gametophyte self-incompatibility in *Nicotiana alata* (Anderson et al., 1986). The optimum time for obtaining the self incompatible response was during anthesis and correlated with the appearance of the S-type glycopeptides. The glycosylated glucanase form of the PR-2,N,O class in tobacco also showed marked accumulation at the time of anthesis. The presence of an abundant (1-3)-β-glucanase activity and the specific localization of the glycosylated PR-2,N,O form in the style, may indicate a role in stylar energy metabolism. However, an endogenous substrate for this glucanase has not been defined. Alternatively, the enzymatic activity may play a part in pollen tube growth, as extracellular glucanases have been shown to function during plant cell elongation (Varner and Liang-Shiou, 1989). Other PR proteins forms accumulate in the flower in a spatially unique manner as has been outlined in the previous section. It was pointed out, that on the background of developmentally timed accumulation, the stylar organ can still show a typical pathogenesis response, namely the concomitant accumulation of the other PR proteins. The complex regulatory pattern may be taken as an indication of the dual functionality of the PR proteins. They play a role in resistance to pathogens and also, as of yet undefined aspects of flower physiology. It is of interest to speculate that plant recognition and reaction to the intrusion of pathogens have some commonality with the penetration of pollen.

4. Conclusion

The discovery of novel developmentally regulated genes has considerably added to the biological significance of these proteins. The ubiquity of PR proteins in plant species also points to their functional importance. We have only recently begun to appreciate the complexity of PR proteins regulation during pathogenesis. However, the physiological and genetic evidence available does not show that the presence of PR proteins by themselves is indicative of a plant's resistance. Most likely, the resistance to pathogens is a result of combined effect of some of the PR proteins and additional defense systems of the plant. The significance of PR proteins in symbiotic associations has only been recently studied. It remains to be seen whether the successful colonization of host plants requires repression of pathogenesis.

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