Local and Systemic Accumulation of Pathogenesis-Related Proteins in Tobacco Plants Infected with Tobacco Mosaic Virus

Thierry Heitz, Bernard Fritig, and Michel Legrand

Institut de Biologie Moléculaire des Plantes du CNRS, Université Louis Pasteur, 12, rue du Général Zimmer, 67084 Strasbourg Cedex, France

Received 12 January 1994. Accepted 3 August 1994.

Tobacco mosaic virus-infected tobacco is a well-defined plant-pathogen system where a set of pathogenesis-related (PR) proteins have been isolated and characterized. We have used a series of seven antisera raised against purified proteins to study, by western blotting, the distribution of four families of PR proteins in leaves of tobacco mosaic virus-infected plants. The highest induction of all the PR proteins was found in a restricted number of cells surrounding the lesions. Differential patterns of accumulation of acidic and basic PR isoforms were detected further from the infection site where systemic acquired resistance is known to develop. Acidic PR proteins were barely detectable between the lesions and were present in variable amounts in upper uninoculated leaves. The basic isoforms, present at low levels in control leaves, were found to be significantly induced both between the necrotic spots on inoculated leaves and at distance in uninoculated parts of inoculated plants.

Additional key words: hypersensitive response, immunodetection, spatial distribution, systemic acquired resistance

In many plants inoculation with certain viruses may lead to the appearence of local necrotic lesions at the site of infection. The necrosis represents a hypersensitive response to the virus which is restricted to the necrotic lesions and the surrounding layer of cells. The leaves bearing lesions produce large amounts of soluble pathogenesis-related (PR) proteins (Van Loon 1985) with acidic or basic isoelectric points (Legrand *et al.* 1987; Kauffmann *et al.* 1987, 1990; Fritig *et al.* 1989; Stintzi *et al.* 1991). In tobacco, acidic PR proteins are localized extracellularly (Parent and Asselin 1984; Van den Bulcke *et al.* 1989; Dore *et al.* 1991), whereas many basic PR proteins have been shown to accumulate inside the cell (Van den Bulcke *et al.* 1989; Van Loon and Gerritsen 1989; Grosset *et al.* 1990; Keefe *et al.* 1990; Dore *et al.* 1991). PR proteins of group 2 and 3 were demonstrated to display β-1,3 glucanase and chitinase activity, respectively (Kauffmann *et al.* 1987; Legrand *et al.* 1987). These enzymes and thaumatin-like proteins (PRs of group 5) have been shown to inhibit fungal growth *in vitro* (Mauch *et al.* 1988; Roberts and Selitrennikoff 1988; Woloshuk *et al.* 1991; Vigers *et al.* 1991, 1992). For some PR proteins, their constitutive expression in transgenic plants was demonstrated to increase resistance to pathogens (Broglie *et al.* 1991; Alexander *et al.* 1993). These findings and the finding that the PR proteins are induced systemically in noninfected tissue may indicate a role for PR proteins in systemic acquired resistance (Ross 1961; Kuc 1982).

It is difficult to compare the patterns of accumulation of the different defense proteins because they have been studied in different plant-pathogen systems. In this work, seven different sera (Legrand *et al.* 1987; Kauffmann *et al.* 1987, 1990; Grosset *et al.* 1990; Stintzi *et al.* 1991) were used which are specific for PR proteins of groups 1, 2, 3, and 5 (for description of groups, see Stintzi *et al.* 1993). These antisera allowed us to demonstrate that the various PR proteins accumulate differentially in the inoculated and non-inoculated leaves of tobacco infected with TMV.

RESULTS

To obtain a complete picture of PR protein accumulation, we used seven specific antisera obtained previously (Legrand *et al.* 1987; Kauffmann *et al.* 1987, 1990; Grosset *et al.* 1990; Stintzi *et al.* 1991) to study the spatial distribution of these proteins in hypersensitively reacting tobacco leaves. The immunospecificity of these antisera is summarized in Table I. In each instance the antisera were PR protein group specific: Group 1 antisera reacted only with group 1 proteins, group 2 antisera only with group 2 proteins and so on. However, within a group there was cross-reactivity with the acidic and basic forms (Legrand *et al.* 1987; Kauffmann *et al.* 1987 1990). For example, antibodies raised against acidic β-1,3 glucanases (PR-2, -N, -O, -Q′) or acidic chitinases (PR-P and -Q) readily recognized basic and acidic members of the same group: antibodies raised against their basic counterparts were specific only for the basic forms. For proteins belonging to group 5, the antibodies obtained against one acidic form (PR-S) and one basic form (osmotin) both cross-reacted with all acidic and basic members of this group (Stintzi *et al.* 1991; Table 1).

Figure 1 presents the immunodetection of PR proteins in extracts of TMV-infected tobacco. The extracts were separated into acidic and basic fractions by ion-exchange chromatography, submitted to electrophoresis, transferred onto nitrocellulose sheets, and finally immunorevealed with the appropriate antiserum. Antisera raised against an acidic or a basic PR isoform were used to analyze the acidic or basic

MPMI Vol. 7, No. 6, 1994, pp. 776-779

protein fraction, respectively. In each case one or several protein bands of the infected leaf extract (Fig. 1, IL 1 and 2) were detected, in agreement with their known reactivity (Table 1). In infected leaves, the acidic PR proteins were strictly confined to disks taken from the necrotic lesions and the closely associated living cells (Fig. 1, IL 1 and IL 2). The strict localization of acidic PR proteins around the infection site was also clearly visible when leaf samples bearing lesions were directly printed onto nitrocellulose sheets (Jung and Hahne 1992; Jung, unpublished results). The acidic proteins were not detected in mock-inoculated leaves (Fig. 1, CL).

In the upper uninoculated leaves there was differential accumulation of the acidic PR proteins: PR-5 proteins were undetectable (Fig. 1, UL); the PR-1 and -3 were present at low levels, whereas fairly high amounts of PR-2 were observed. We never detected any acidic PR protein in extracts of leaves above water-inoculated controls (CL*, acidic). It is noteworthy that, in the case of acidic PR-2 and -3 proteins, the induction was more pronounced in upper leaves than between the necrotic lesions of an inoculated leaf (compare UL to IL 2 in Fig. 1).

The basic proteins were present at significant background levels on blots of control leaves. This is in accordance with the previously reported constitutive expression of these proteins in the oldest leaves of healthy tobacco (Felix and Meins 1986; Shinshi *et al.* 1987). In the inoculated leaves the basic forms, like the acidic forms, accumulated to highest levels in and around the lesions. However, unlike the acidic forms, these proteins were also induced to higher levels than in the mock-inoculated leaf in the regions between lesions. On blots of upper leaves (UL), a low level of induction was observed for basic glucanase (PR-2), whereas marked increases in the level of basic proteins of groups 3 and 5 were detected. In the case of basic chitinases the western blots disclosed the induction of 3 reactive proteins while only one band was detected in the corresponding control (CL*).

DISCUSSION

The spatial distribution of endogenous PR gene products in TMV-infected tobacco plants has been investigated by using seven different antisera with well-defined specificity. For all PR proteins detected in this analysis, a maximum accumulation was observed in a limited number of cells surrounding the infection site. These data therefore confirm and extend to four serological groups of PR proteins the earlier findings of Antoniw and White (1986) who used ELISA to show that PR-1 accumulates in and around the necrotic zone of TMV lesions.

We have previously shown that the virus is restricted to necrotic areas and to a narrow ring of living cells surrounding

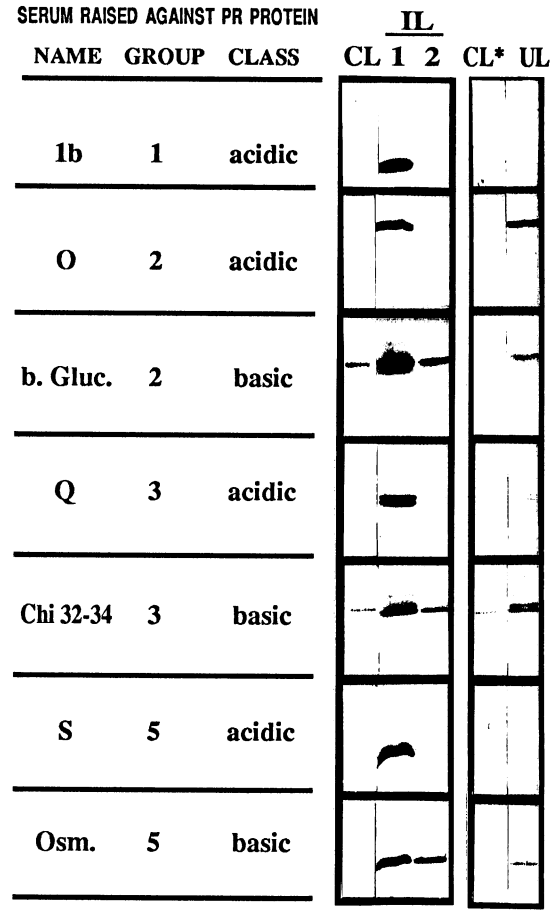

Fig. 1. Distribution of tobacco PR proteins in inoculated and uninoculated leaves of TMV-infected plants. Plant tissue was harvested 7 days after TMV inoculation. Total leaf proteins were extracted, separated in acidic and basic fractions and blotted. The blots were probed with specific sera (see Table 1) whose group and subclass (acidic or basic) are indicated. CL: control leaves. IL: infected leaves which were divided in two samples for blot analysis, a 7-mm-diameter disk centered on the necrotic spots (IL 1) and the tissue situated between the lesions (IL 2). UL : upper non-infected leaves of infected plant. CL* : upper control leaves of non-infected plant.

Table 1. Serological relationships between the major PR proteins of tobacco[a]

Purified PR proteins			Antisera raised against PR						
Group	Name	Class	1b	O	b. gluc.	Q	chi 32, 34	S	osm.
1	1a, 1b, 1c	II (acidic)	+++	–	–	–	–	–	–
2	b, gluc,	I (basic)	–	++	+++	–	–	–	–
	2, N, O	II (acidic)	–	+++	–	–	–	–	–
	Q´	III (acidic)	–	+++	–	–	–	–	–
3	chi 32-34	I (basic)	–	–	–	++	+++	–	–
	P,Q	II (acidic)	–	–	–	+++	–	–	–
5	osmotin	I (basic)	–	–	–	–	–	+	+++
	R,S	II (acidic)	–	–	–	–	–	+++	++

[a] The immunological relationships were studied by western blotting 0.1 µg of each purified protein. All the purified proteins were tested with each antiserum raised against one representant of the acidic and basic PR subclass. +++ : strong reactivity of homologous proteins. ++ and + : crossreactivity with proteins of an other subclass. – : no reaction. b. gluc : basic glucanase. chi 32-34: 32 kDa and 34 kDa basic chitinases.

the lesions (Legrand *et al.* 1976). This implies that PR protein induction observed in distant tissues is mediated by secondary signal(s), one of the candidates being salicylic acid (for review, see Malamy and Klessig 1992; Gaffney *et al.* 1993). In the present study, the induction in the tissue between the lesions and in upper leaves were compared. The relative induction of the different proteins was found highly variable: acidic PR-5 were not induced outside the stimulated ring around the lesion, whereas others are clearly induced at larger distance. Acidic glucanases (PR-2) and basic chitinases (PR-3) accumulated at a higher level in uninoculated leaves of infected plants than in cells situated outside of the lesions on the inoculated leaf. These observations suggest that PR genes are affected differently by the regulatory signal(s), and even a given gene may be induced at various levels according to the leaf position. Whether this latter situation arises from distinct signaling systems at short and long distance from the infection site, or is due to fine tuning of the same signal remains unknown.

The synthesis of PR proteins has often been associated with systemic acquired resistance which is induced in uninfected parts of tobacco reacting hypersensitively to TMV (Kassanis *et al.* 1974; Van Loon and Gerritsen 1989). Systemic acquired resistance results in a decrease in symptom severity from secondary infection by the same or different pathogens (Ross 1961; Kuc 1982). Major acidic PR proteins have been detected in uninfected parts of TMV-infected tobacco (Van Loon and Gerritsen 1989), but the pattern of mRNA accumulation was found very different in uninoculated leaves between the genes for the acidic and basic PR proteins: Messengers encoding acidic isoforms were found to accumulate in upper leaves of TMV-infected plants, whereas for genes encoding basic proteins, only expression of some members (Ward *et al.* 1991), or no expression at all (Brederode *et al.* 1991) was detected. This low level of steady-state messengers for basic isoforms which accumulate systemically in response to TMV infection, may indicate that the synthesis of those PR proteins is not correlated to the level of mRNAs in a simple manner. Furthermore, PR proteins are known to have a low turnover, and their accumulation could arise from almost undetectable mRNA levels. Thus, several molecular mechanisms may control the expression of the PR genes during the local hypersensitive response and during the development of the systemic acquired resistance.

MATERIALS AND METHODS

Plant material.

Tobacco (*Nicotiana tabacum* 'Samsun NN') plants were grown in a greenhouse under controlled conditions. Three fully expanded leaves were inoculated with a suspension of purified TMV, or mock-inoculated with water. After incubation for 7 days in a growth chamber at 22° ± 1° C under a photoperiod of 16 hr, the material was harvested in five samples as follows: 1) 7-mm-diameter disks including the necrotic lesion and a ring of living cells were excised with a cork borer, 2) tissue between the lesions was collected, 3) the second leaf above the youngest infected leaf was harvested for the study of systemic induction, 4) and 5) on mock-inoculated plants, healthy leaves of the same size as those from infected plants (inoculated leaf and the second leaf above) were used as controls. All samples were collected from at least three plants.

Extraction and fractionation of protein.

Five grams of fresh material was ground with pestle and mortar in one volume of 0.5 M sodium acetate, pH 5.2, containing 15 mM β-mercaptoethanol and 50 mg of charcoal. The homogenate was centrifuged at 10,000 *g* for 30 min. The supernatant was desalted on a prepacked Sephadex G_{25} column (PD 10, Pharmacia) equilibrated with 20 mM sodium acetate, pH 5.2. The protein fraction was kept at 4° C for several hours. The clear supernatant obtained after centrifugation at 10,000 *g* for 30 min was fractionated on a S-Sepharose column (1.3 × 5.5 cm, Fast Flow, Pharmacia) equilibrated with 2 mM sodium acetate, pH 5.2. The non-adsorbed fraction contained acidic proteins, whereas basic proteins were eluted with 20 mM sodium acetate, pH 5.2, containing 0.5 M NaCl. The two fractions were concentrated on Centricon 10 ultrafiltration membranes (Amicon).

Blotting experiments.

Proteins were separated on SDS-polyacrylamide gels, blotted onto nitrocellulose and probed with specific sera, as described by Geoffroy *et al.* (1990). A serum raised against an acidic or a basic PR isoform was used to analyze the acidic or basic protein fraction, respectively. The antisera have been obtained as described previously (Kauffmann *et al.* 1987; Legrand *et al.* 1987; Kauffmann *et al.* 1990; Stintzi *et al.* 1991).

ACKNOWLEDGEMENTS

We thank J. L. Jung for performing tissue printing experiments.

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