106. *Precise Mode of Induction of Pathogenesis-related Proteins by Chemical Treatment in Tobacco*

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Introduction. Pathogenesis-related proteins (PR proteins) accumulate as novel soluble proteins in various plants when they are infected and react hypersensitively to viruses (Van Loon and Van Kammen, 1970; Redolfi, 1983), bacteria (Ahl *et al.*, 1981) and fungi (Gianinazzi *et al.*, 1980). The production of the proteins is also induced by chemical treatment such as with salicylic acid (White, 1979) or eosin yellowish (Ohashi and Matsuoka, 1985). Recently, we demonstrated the induction of PR proteins by some stresses, e.g., cutting, injury or osmotic stress (Ohashi and Matsuoka, 1985). The role of these proteins is not yet clear, but leaves containing a significant amount of PR proteins are always more resistant to infection by pathogens than those which only contain little. These proteins are not degradative products from pre-existing larger precursors but accumulate from net protein synthesis (Matsuoka and Ohashi, unpublished data). Therefore, PR proteins may have any roles in plant resistance to pathogens or stresses under abnormal conditions. The time course of the accumulation of these PR proteins must be elucidated to clarify their functions.

This paper describes the precise mode of induction of PR proteins in tobacco leaves by chemical treatment using quantitative determination by application of rocket immunoelectrophoresis with antibody of PR1a, which is the most acidic of the PR proteins.

Materials and methods. Tobacco plants (*Nicotiana tabacum*, cv. Samsun NN) were grown in a temperature-controlled greenhouse (20–30°C) for 2 to 3 months after sowing. Fully expanded leaves were detached, and seven discs, 19 mm in diameter, were punched out. These discs were inoculated with TMV and incubated on wet filter paper, or floated on 10 ml of water or a solution containing various concentrations of potassium salicylate or eosin yellowish in a Petri dish (9 cm in diameter), and kept at 20°C with constant illumination of 6,000 lux. For the time course analysis, discs were ground with a chilled mortar and pestle in 84 mM citrate-32 mM sodium phosphate buffer, pH 2.8, and the content of PR proteins in the supernatant after centrifugation for 15 min at 8,000×g was determined by rocket immunoelectrophoresis using anti PR1a Immunoglobulin G (IgG) as described by Ohashi and Matsuoka (1985).

Double immunodiffusion tests were carried out as described by Ouchterlony and Nilsson (1973) on a 0.8% agarose plate.

Electrophoresis was performed on 10% polyacrylamide slab gels as described by Davis (1964).

Results and discussion. Fig. 1 shows the results of double immunodiffusion tests of PR proteins induced in TMV-infected or chemical-treated tobacco leaves. There was only the universal precipitin line without any spurs between the crude

Fig. 1. Double immunodiffusion tests of crude extracts from TMV-infected or chemical-treated Samsun NN tobacco leaves. Center well, anti-PR1a IgG (250 μ g); well 1, crude extract from 0.2 mg of TMV-infected leaves; well 2, crude extract from 0.2 mg of potassium salicylate-treated leaves; well 3, crude extract from 0.2 mg of eosin yellowish-treated leaves.

extract from TMV-infected leaves and salicylate- or eosin yellowish-treated leaves against anti PR1a IgG.

Previous studies (Matsuoka and Ohashi, 1984; Ohashi and Matsuoka, 1985) showed that the main PR proteins (PR1a, PR1b and PR1c) from TMV-infected or salicylate-treated tobacco leaves have similar molecular weights and are identical in their immunoreactivity to the antibody in agarose gel. Also rocket immunoelectrophoresis using anti PR1a has been shown to be effective as a sensitive immunological method for quantitative determination of PR proteins from these leaves (Ohashi and Matsuoka, 1985).

Electrophoresis showed that PR-proteins induced by eosin yellowish were almost the same qualitatively as those induced by TMV-infection or potassium salicylate in 5 days (Fig. 2). Accordingly, PR proteins from the leaves treated with eosin yellowish, as well as those induced by TMV infection or potassium

Fig. 2. Polyacrylamide gel electrophoresis of PR proteins induced by potassium salicylate, eosin yellowish and TMV-infection. Crude extracts from 20 mg of water-treated leaf (lane 1), potassium salicylate-treated leaf (lane 2), eosin yellowish-treated leaf (lane 3) and TMV-infected leaf (lane 4).

salicylate, could be determined quantitatively by rocket immunoelectrophoresis.

Therefore, the increment of PR proteins at 5 days after chemical treatments was studied by this quantitative method. Table I shows the induced amounts of PR proteins by eosin yellowish and potassium salicylate. The dose responses were observed with both chemicals.

Table I. Induction of PR proteins by chemicals on discs of Samsun NN tobacco leaves in 5 days

Chemical	Concentration (mM)	PR proteins $\mu\text{g/g}$ fresh weight
Potassium salicylate	0.14	22.8
	0.36	92.5
	0.72	111.0
	1.44	120.0
Eosin yellowish	0.002	40.5
	0.01	108.6
Water	—	0.5

The leaf discs were incubated in solutions containing various concentrations of these chemicals. Crude extract of these discs was prepared as described in Materials and methods. Contents of PR proteins were determined by rocket immunoelectrophoresis.

But eosin yellowish was much more 70-fold effective than potassium salicylate, in fact even a low concentration such as $10 \mu\text{M}$ was sufficient to induce large amounts of PR proteins. Most of the effective inducers of PR proteins reported are derivatives of salicylic acid (2-hydroxybenzoic acid). And eosin yellowish is also a derivative of salicylic acid.

Rocket immunoelectrophoresis was also applied to determine the mode of induction of PR proteins by chemical treatments (Fig. 3).

The induction kinetics by either chemicals were very similar, and the amount of PR proteins started to increase at 2 days after treatment and reached maximum at 5 to 7 days. The accumulated amounts exceeded $100 \mu\text{g}$ per g fresh leaf at

Fig. 3. Induction of PR proteins after treatment with potassium salicylate or eosin yellowish. Samsun NN tobacco leaves were incubated on water (○), 0.72 mM solution of potassium salicylate (●) or $10 \mu\text{M}$ solution of eosin yellowish (▲).

7 days. The two chemicals produce more than 200-fold increase of these proteins compared with the water-treated control discs (Table I). As the content of the proteins in healthy leaves was 100 ng or less per g fresh weight, determining from the concentrated protein solution of healthy leaf extract, the net amount of these proteins induced by the chemicals in 5 days reached more than 1000-fold. What role do PR proteins have to lead to such a large increase in their amount? Eosin yellowish treatment of Samsun NN tobacco or bean leaves induced obvious resistance to TMV multiplication or cell-to-cell transfer according to the measurement of local lesion development by TMV (Shimomura, 1976). Tobacco leaf treated with salicylic acid also resisted TMV-multiplication on systemic infection and on local infection (Shimomura and Ohashi, 1984). As PR proteins always increased in high level in these cases, acquisition of defence mechanism against the development of virus disease in these tissue may depend on the presence of PR proteins suggesting they have a function to resist to pathogens.

A very small amount of PR proteins was formed in the control discs and increased with time (Fig. 3). However, the degree of the increment by cutting stress was two orders lower than those induced by chemical treatment (Table I). These proteins are also induced by some kinds of stress not only cutting, but also injury or hypertonic treatment (Ohashi and Matsuoka, 1985). As they were synthesized with net protein synthesis (Matsuoka and Ohashi, unpublished data), these proteins may have some roles in stress conditions.

To study the function of these proteins, further experiments must be conducted such as on their localization in cell or tissue, and on the time course of their synthesis of *in vivo* and time course of synthesis of their m-RNA. We are now working to find the answers to these questions.

This paper is the first report to elucidate the precise mode of induction of PR proteins by potassium salicylate and by eosin yellowish, and to clarify the net increase of these proteins by quantitative determination using immunological methods.

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