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The chemical induction of PR (b) proteins and resistance to TMV infection in tobacco

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Summary

Aspirin injected into Xanthi-nc tobacco leaves induces the production of PR protein and resistance to TMV. The concentration of PR protein and resistance increases with increasing aspirin concentration, up to a plateau. 2-Thiouracil and dioxohexahydrotriazine also induce PR protein when injected into tobacco leaves. Barium and manganese salts induced PR protein, but those of eight other cations did not. Certain salts were phytotoxic but did not induce PR protein, confirming that the production of PR protein is not a non-specific stress response.

PR protein; TMV; *Nicotiana tabacum*; resistance

Introduction

Infection of *Nicotiana tabacum* cvs Xanthi-nc and Samsun NN with tobacco mosaic virus (TMV) is followed by the appearance of necrotic lesions at the sites of infection and the production of new soluble leaf proteins [9,14] called pathogenesis related (PR) proteins [2] or b proteins [9]. These proteins are produced by the plant in response to infection and may be involved in the localisation of the virus to the lesion and increased resistance to further infection which is induced at the same time. Both PR proteins and resistance to virus infection are induced in tobacco when leaves are treated with certain chemicals, e.g. polyacrylic acid [10], ethephon [13], salicylic acid and related compounds [15], and some plant growth substances [4].

In this paper we report the effects of the antiviral chemicals 2-thiouracil [7], dioxohexahydrotriazine (DHT) [12], and a range of inorganic salts on PR protein induction. We also report the effects of treating leaves with different concentrations of

acetylsalicylic acid on the induction of PR protein and resistance to virus infection.

Materials and Methods

Plant material

Nicotiana tabacum cultivars Xanthi-nc and Samsun NN were grown in 13 cm diameter pots using a sand-peat compost, in a glasshouse at 20–25°C with supplementary light during winter.

Injected chemicals

Dioxohexahydrotriazine (DHT) was a gift from Professor G. Schuster (Karl-Marx-University, Leipzig, G.D.R.). Solutions of aspirin (acetylsalicylic acid), 2-thiouracil and DHT were adjusted to pH 6.5 before injection. The solutions of metal salts were injected without adjusting pH because some of the salts were not soluble at pH 6.5. Solutions were injected near lateral veins in the lower leaf surface so that the intercellular leaf spaces of the entire leaf were infiltrated. Approximately 0.5 ml solution was injected per gram of leaf. Seven days later treated leaves were harvested and PR proteins extracted. Resistance to virus infection was measured 7 days after treatment by inoculating with 0.5 μg/ml TMV and measuring lesion size and/or number 7 days later. Lesion size was measured by using a Leitz microscope connected to a Quantimet 720 image analysing computer. At least 10 lesions were measured on each half leaf.

Polyacrylamide gel electrophoresis

Samples of 5 g of leaf were homogenized in 5 ml 84 mM citric acid, 32 mM Na_2HPO_4, 14 mM 2-mercaptoethanol and 6 mM L-ascorbic acid at a final pH of 2.8. The homogenate was centrifuged for 5 min at 8000 × *g* and the supernatant applied to a column (25 × 2.5 cm) of Sephadex G50 (fine) equilibrated in 50 mM Tris-HCl, 1 mM EDTA (Tris buffer) final pH 8.0 at 25°C. Fractions of 10 ml were collected and those containing protein, as judged by their absorbance at 280 nm, were pooled and concentrated by freeze-drying. The samples were redissolved in 1 ml distilled water and dialysed against gel-running buffer before analysis by PAGE. Samples were analysed in 10% (w/v) acrylamide gels at pH 8.9 as described by Ornstein [11]. After electrophoresis the bromophenol blue front was marked and the gels stained with Coomassie brilliant blue G250 as described previously [1]. After storage for at least 2 days in 5% acetic acid the gels were scanned using a Beckman model 25 spectrophotometer. The PR-1a protein, identified by its $R_f = 0.81 \pm 0.01$, was then estimated by measuring its peak height.

Enzyme-linked immunosorbent assay (ELISA)

The indirect ELISA procedure using $F(ab')_2$ fragments of immunoglobulin was as described by Barbara and Clark [6]. The procedure to determine PR-1a and TMV concentrations in leaf extracts was as described by Antoniw et al. [5] except that the polystyrene microtitre plates used were Nunc Immuno Plate II. Leaf samples (1 g) were

extracted in 4 ml of phosphate-buffered saline containing 0.5 ml/l Tween 20, 20 g/l polyvinylpyrrolidone (mol. wt. 44 000) and 2 g/l ovalbumin. Each sample was assayed in duplicate as six 10-fold serial dilutions in the same buffer. A standard of purified PR-1a [1] was included on each microtitre plate with four samples. Sample concentrations were estimated from the standard on the same plate by plotting absorbance against log standard concentration and fitting a logistic curve.

Results

The induction of PR proteins and resistance by different concentrations of aspirin

Solutions of aspirin from 9 to 600 μg/ml were injected into healthy Xanthi-nc leaves and 7 days later the leaves were harvested. At the two highest concentrations (600 and 300 μg/ml) white necrotic patches were apparent, probably due to a phytotoxic effect of aspirin. PR-protein and resistance measurements were made only at a series of lower concentrations where there was no apparent phytotoxicity (Fig. 1). The amount of PR-1a (b_1) induced increased with the concentration of aspirin injected, and then reached a plateau. The size and number of TMV-induced lesions decreased with increasing aspirin concentration, but lesion size was more sensitive than lesion number which only began to decrease at 75 μg aspirin/ml. In general this showed that, as the dose of aspirin increased, so did the amount of PR-1a produced and the resistance to TMV. Analysis of variance showed clear differences ($P < 0.001$) between lesion sizes, transformed to square roots, induced by TMV in leaves pretreated with different concentrations of aspirin. A linear regression of lesion size on concentration of aspirin was significant ($P < 0.001$) and demonstrated that lesion size was approximately halved when aspirin concentration was doubled.

In another experiment the amounts of PR protein produced in Xanthi-nc, 7 days

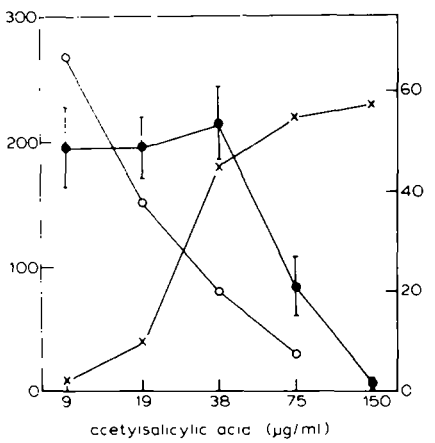

Fig. 1. The effect of aspirin on PR-1a (b_1) protein induction and TMV lesion size and number. ○——○, lesion area ($mm^2 \times 10$); ×——×, b_1 peak height from gel scans (mm); ●——●, lesion number, variation in data shown by standard error bars.

after injection with a range of aspirin concentrations, were measured by $F(ab')_2$ ELISA (Fig. 2). This showed the same pattern with less than 50 ng PR-1a/g leaf in healthy leaves and a plateau of about 50 μg PR-1a/g leaf in leaves injected with the highest levels of aspirin. In other experiments as much as 140 μg PR-1a/g leaf has been induced in Xanthi-nc leaves 7 days after injection of 150 μg aspirin/ml.

Induction of PR proteins by other chemicals

Inorganic salts. A range of inorganic salts at concentrations from 1 to 100 mM were injected into Xanthi-nc leaves. 7 days later the appearance of the injected leaves was noted and leaf extracts were analysed for PR proteins (Table 1). Leaves injected with water were used as a control and leaves injected with the known inducers of PR proteins, aspirin and polyacrylic acid, were included for comparison. $CuCl_2$ and $HgCl_2$ were both phytotoxic at 1 mM so the presence of PR proteins was not tested. Two of the salts, $BaCl_2$ and $MnCl_2$, induced PR proteins (Fig. 3) and like aspirin and polyacrylic acid, they also caused chlorosis of the injected leaf. However, other salts also produced chlorosis ($FeSO_4$ produced chlorosis similar to that induced by polyacrylic acid) but did not induce PR proteins. Pricking leaves with ethephon, HCl or H_3PO_4 gives rise to necrosis but only ethephon induces PR proteins [13]. Thus PR proteins appear not to be induced by general stress or by reaction to a phytotoxic effect of the inducer, but are a response to more specific stimuli. The induction of PR proteins by $BaCl_2$ is temperature-sensitive and does not occur at 40°C (data not shown).

Seven days after injecting leaves with a range of $MnCl_2$ concentrations PR protein was measured by $F(ab')_2$ ELISA (Table 2). The amounts of PR protein induced

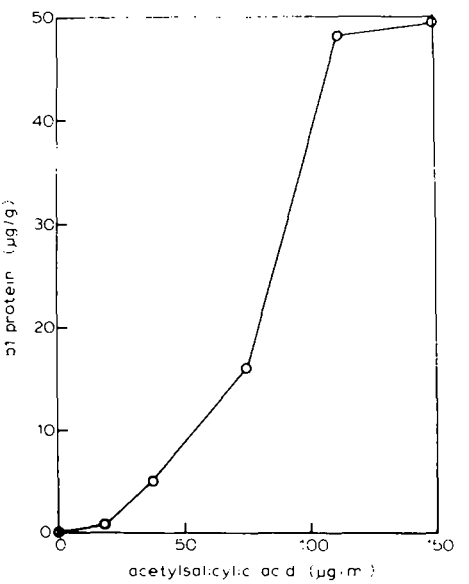

Fig. 2. PR-1a (b_1) protein accumulation in cv. Xanthi-nc leaves 7 days after injection with a range of aspirin concentrations. Each treatment was duplicated, differences between duplicates were smaller than the symbols.

TABLE 1

The induction of PR proteins and symptoms produced in Xanthi-nc leaves injected with various inorganic salts

Treatment		Presence of PR proteins	Symptom of injected leaf
Drug	Conc. (mM)		
$BaCl_2$	1	+	slight chlorosis
	10	+	chlorosis + 25% leaf dried out
$CaCl_2$	30	-	normal
	100	-	chlorosis
$CoSO_4$	1	-	chlorosis + necrotic areas
$CuCl_2$	1	ND	90% dried out
$FeSO_4$	1	-	chlorosis
$HgCl_2$	1	ND	dried out
$MgCl_2$	30	-	normal
	100	-	chlorosis
$MnCl_2$	20	+	chlorosis + small dark necrotic patches
$SnCl_2$	1	-	slight chlorosis
$ZnSO_4$	1	-	chlorosis
Healthy	-	-	normal
Aspirin	1	+	chlorosis
Polyacrylic acid	0.05	+	chlorosis

ND = not determined.

increased with increasing concentration of $MnCl_2$ injected but were well below the level induced by aspirin. Like aspirin, but unlike polyacrylic acid [3], manganese salts induced PR proteins in cv. Samsun NN. $Mn(NO_3)_2$ and $MnSO_4$ were as effective as $MnCl_2$ at inducing PR proteins, $MnCO_3$ was not as effective, probably due to its low solubility, and manganese acetate was also not as effective, probably because it is phytotoxic (Table 3).

2-Thiouracil. 7 days after injection with a solution of 100 μg 2-thiouracil/ml leaves of Xanthi-nc and Samsun NN contained large amounts of PR-1a (b_1) and 1b (b_2) (Fig. 3). Leaves of both cultivars inoculated with TMV 7 days after injection with 100 μg 2-thiouracil/ml were immune from infection.

Dioxohexahydrotriazine (DHT). Schuster et al. [12] showed that spraying tobacco plants with DHT 2 days before inoculation and subsequently 2 and 7 days after inoculation, significantly decreased the concentration of potato viruses X and Y, and cucumber mosaic virus. However, seven days after spraying cv. Xanthi-nc leaves with a solution of 1.25 μg DHT/ml no increase in PR proteins was detectable but injecting DHT did have an effect (Table 4). At 1.25 μg DHT/ml there was a 2–3-fold increase of PR protein over the control but at 10 μg/ml DHT this became 180–190-fold.

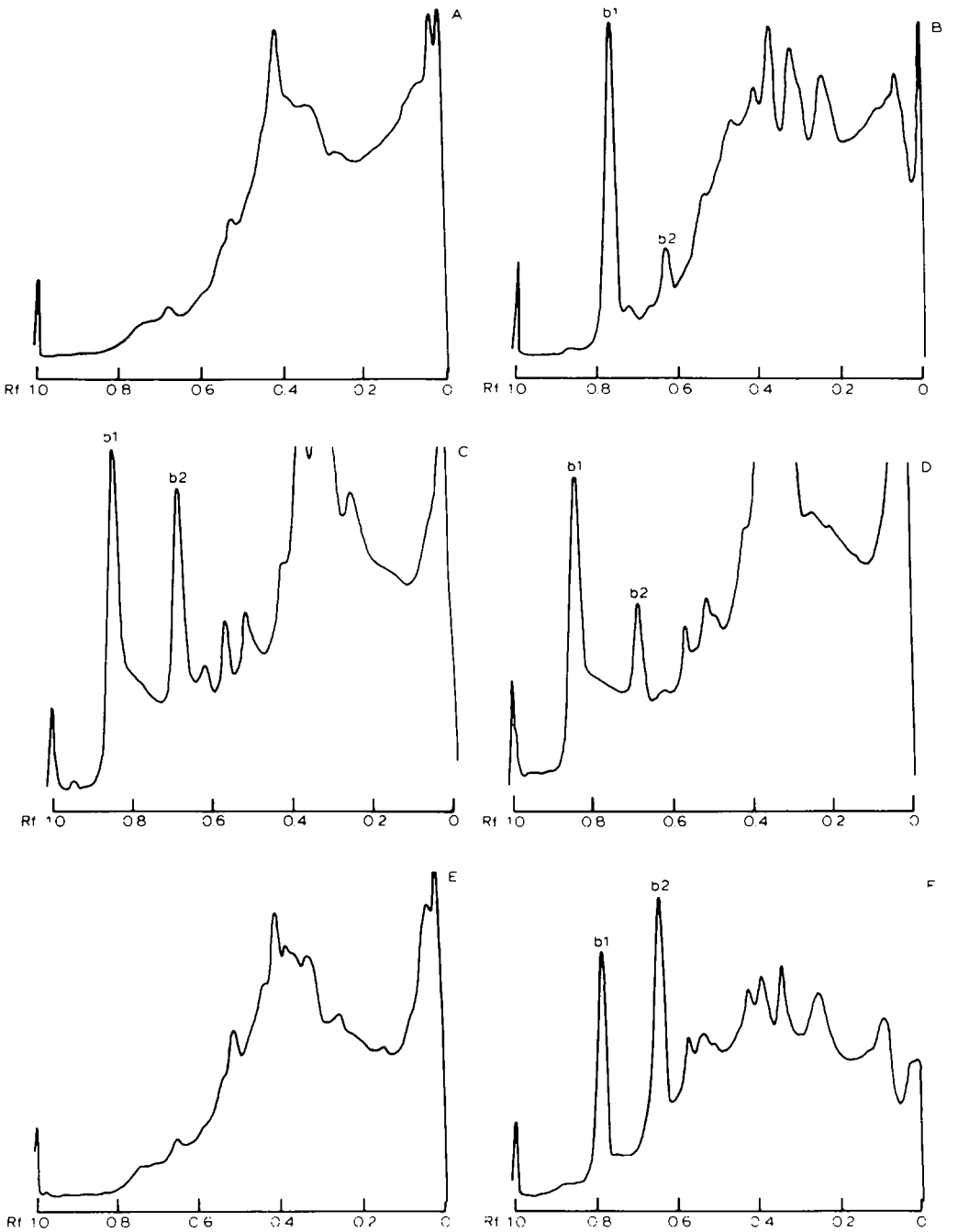

Fig. 3. Gels scans after electrophoresis on 10% polyacrylamide non-denaturing gels of extracts from Xanthi-nc (A, B, C, D) and Samsun NN (E, F) leaves injected 7 days previously with distilled water (A, E), 100 μg 2-thiouracil per ml (B, F), 10 mM $BaCl_2$ (C) and 40 mM $MnCl_2$ (D).

TABLE 2

Amounts of PR protein induced by a series of $MnCl_2$ concentrations

Treatment		PR protein[a] (ng/g leaf)	
Drug	Conc. (mM)	Xanthi-nc	Samsun NN
$MnCl_2$	40	12900	1217
	30	ND	1002
	20	1230	ND
	10	269	ND
	5	22	ND
Control	-	40	26
Aspirin	1	138000	41300

[a] Determined by ELISA.
ND = not determined.

TABLE 3

The induction of PR protein by different manganese salts in cv. Xanthi-nc

Treatment		PR protein[a] (ng/g leaf)
Drug	Conc. (mM)	
Mn acetate	40	760[b]
$MnCl_2$	40	15000
$MnCO_3$	?[c]	640
$Mn(NO_3)_2$	40	9080
$MnSO_4$	40	10300
Control		110
Aspirin	1	60000

[a] Determined by ELISA.
[b] Phytotoxic.
[c] Low solubility; therefore used as saturated solution.

Discussion

The good correlation between the concentration of aspirin injected and the reduction in lesion size suggests that PR proteins are very closely associated with the mechanism responsible for reduction in lesion size (when aspirin is the inducing agent); lesion size was decreased by concentrations of aspirin less than those necessary to decrease lesion number. Lesion size seems more sensitive than lesion number as an indicator of resistance.

The antiviral effects of DHT and 2-thiouracil may be not only as RNA base analogues interfering with nucleic acid metabolism, but also by inducing the resistance mechanism associated with PR proteins.

TABLE 4

The induction of PR protein in Xanthi-nc leaves injected or sprayed with DHT

Treatment		PR protein[a] (ng/g leaf)
Formula	Conc. (μg/ml)	
Injection[b]	0	22.3
	1.25	59
	2.5	91
	5	421
	10	4210
Spray[c]	0	8.5
	1.25	7.1

[a] Determined by ELISA.
[b] PR proteins determined on day 7 post injection.
[c] Leaves were sprayed with DHT until run-off on days 0 and 5; PR proteins were determined on day 10.

Two of the range of inorganic salts (barium chloride and manganous chloride) induced PR proteins (but not to the levels stimulated by aspirin). Various salts of manganese were effective, showing that it is the cation which is responsible for PR protein induction. Injection of inorganic salts at these concentrations stresses the leaves and causes the appearance of chlorosis, but chlorosis was not a good indicator of the induction of PR proteins. Thus PR protein induction is not part of a general response to stress, but a specific response induced by certain forms of stress and is closely associated with virus localisation.

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