The EMBO Journal vol.4 no.9 pp.2167–2171, 1985

Virus-induced synthesis of messenger RNAs for precursors of pathogenesis-related proteins in tobacco

R.A.M.Hooft van Huijsduijnen, B.J.C.Cornelissen, L.C.van Loon[1], J.H.van Boom[2], M.Tromp[2] and J.F.Bol

Department of Biochemistry, State University of Leiden, Wassenaarseweg 64, and [2]Department of Organic Chemistry, State University of Leiden, Wassenaarseweg 76, 2333 AL Leiden, and [1]Department of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

Communicated by L.Bosch

Infection of Samsun NN tobacco with tobacco mosaic virus (TMV) induces a number of host-encoded, so-called pathogenesis-related (PR-) proteins, which are found in the intercellular space of the leaf and are associated with induced resistance. By immunoprecipitation of their *in vitro* translation products we were able to detect the mRNAs corresponding to a number of PR-proteins in TMV-infected tobacco, but not in healthy plants. Analysis by the Northern blot technique using cloned cDNA of PR1-mRNAs as probe showed that the mRNAs for the closely related proteins PR1a, 1b and 1c occur at a low level in healthy tobacco; upon TMV infection this level is increased >100-fold. The PR1-specific probe did not hybridize to mRNAs corresponding to other PR-proteins. Sequencing of the 5′-terminal region of PR1-mRNAs showed that PR1-proteins are derived from precursors by removal of an N-terminal signal peptide of 30 amino acids.

Key words: gene family/pathogenesis-related proteins/signal peptide/TMV infection/tobacco

Introduction

Infection of a number of plant species with viruses, viroids, fungi or bacteria induces the accumulation of pathogenesis-related proteins (PR-proteins). This induction has been studied in most detail in *Nicotiana tabacum* cultivars (for a recent review, see van Loon, 1985). As the appearance of PR-proteins is closely associated with the phenomenon of 'systemic acquired resistance' an antiviral function has been suggested for them (Kassanis *et al.*, 1974; van Loon, 1975), even though resistance develops before detectable accumulation of PR-proteins (Fraser, 1982). The PR-proteins induced in tobacco cultivars Samsun NN or Xanthi-nc by infection with tobacco mosaic virus (TMV) or chemical treatment can be separated into >10 components by electrophoresis in non-denaturing polyacrylamide gels (van Loon, 1975, 1982; Antoniw *et al.*, 1980; Pierpoint, 1983). The most predominant proteins are PR1a (IV, b1), PR1b (III, b2), PR1c (II, b3), P, Q and S (the notation given between parentheses is no longer commonly used). These proteins share a number of properties: they are selectively extracted at low pH (van Loon, 1975; Gianinazzi *et al.*, 1977), they occur mainly in the intercellular spaces of the leaf (Wagih and Coutts, 1982; Parent and Asselin, 1984) and they show resistance to a variety of proteases (van Loon, 1982; Pierpoint, 1983). By hydrodynamic measurements, a mol. wt. of 14 200 has been determined for the proteins PR1a, 1b and 1c (Antoniw *et al.*, 1980). Moreover, PR1a, 1b and 1c are related by amino acid composition (Antoniw *et al.*, 1980) and by serology (Matsuoka and Ohashi, 1984; Antoniw *et al.*, 1985).

Carr *et al.* (1982) have reported that the mRNAs for the PR1-proteins and presumably also PR2 are present in equal amounts in healthy and TMV-infected Xanthi-nc tobacco, suggesting that synthesis of PR-proteins is controlled at the level of translation. It was claimed that the non-polysomal mRNAs for PR-proteins in healthy plants are associated with four polypeptides that are removed upon virus infection (Carr, 1983).

We undertook to investigate further the induction of PR-proteins and their mRNAs by TMV infection of Samsun NN tobacco. Antisera raised to a mixture of PR-proteins and to purified PR1a, 1b and 1c were used to identify PR-mRNAs by immunoprecipitation of their *in vitro* translation products. Information on the N-terminal amino acid sequence of PR1a (J.Lucas, personal communication) was used to construct DNA primers that allowed sequencing of the 5′-terminal region of PR1-mRNAs. Moreover, these primers were used to identify cDNA clones of PR1-mRNAs in a cDNA library of poly(A) RNA from TMV-infected tobacco, and these clones were used to analyse PR1-mRNAs in healthy and TMV-infected tobacco by the Northern blot technique.

Results

Figure 1 shows an SDS-polyacrylamide gel run with a mixture of PR-proteins (lane D); to identify the respective PR-proteins in our gel system, purified components were run in parallel lanes. In contrast to the results of others (Matsuoka and Ohashi, 1984), PR1a migrates somewhat slower than PR1b and 1c in our gel

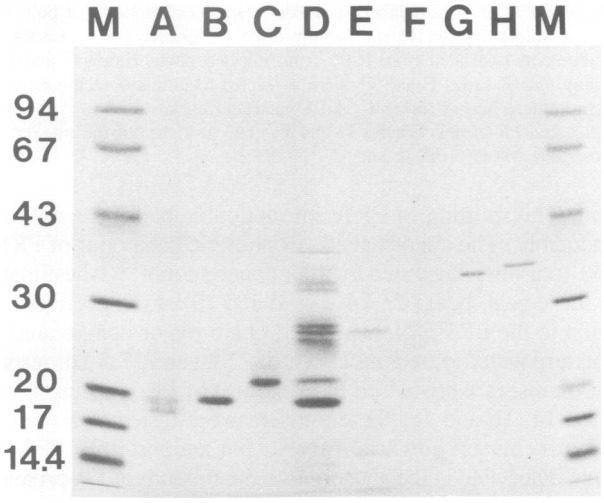

Fig. 1. SDS-PAGE in a 12% gel of PR-proteins from TMV-infected Samsun NN tobacco. The lanes were loaded with PR1c (**lane A**), PR1b (**lane B**), PR1a (**lane C**), unfractionated PR-proteins (**lane D**), P (**lane E**), Q (**lane F**), PR2a (**lane G**) and PR2b (**lane H**). The mol. wts. of marker proteins (**lanes M**) are given in kd. The gel was stained with Coomassie brilliant-blue.

Table I. Serological relationship between PR-proteins

Antiserum	PR-proteins tested							
	All PRs	1a	1b	1c	P	Q	2a	2b
Pre-immune	–	–	–	–	–	–	–	–
Anti-all PRs	+	+	+	+	+	+	–	–
Anti-PR1a	+	+	+	+	–	–	–	–
Anti-PR1b	+	+	+	+	–	–	–	–
Anti-PR1c	+	+	+	+	–	–	–	–

Serological reactions were tested by the Ouchterlony technique; plus signs indicate visible precipitation bands, minus signs indicate that no precipitation was observed. Antisera were used undiluted or in 2-fold dilutions; PR-proteins were tested at a concentration of 1 mg/ml.

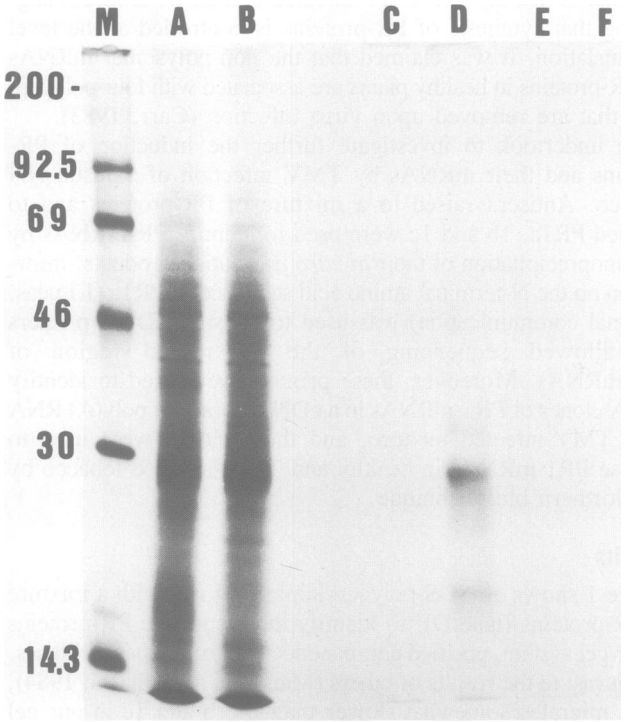

Fig. 2. SDS-PAGE of radiolabelled *in vitro* translation products of poly(A) RNA from infected (**lane A**) and healthy (**lane B**) tobacco plants. **Lanes C–F**: proteins translated from RNA from infected plants (**lanes C** and **D**) or healthy plants (**lanes E** and **F**) were subjected to immunoprecipitation with pre-immune serum (**lanes C** and **E**) and antiserum raised to unfractionated PR-proteins (**lanes D** and **F**). The mol. wt. of the marker proteins (**lane M**) is given in kd.

system. The splitting of PR1c into a double band was not seen reproducibly. The abnormal electrophoretic behaviour of PR1a, 1b and 1c is also illustrated by their apparent mol. wts. estimated from these gels, being 21 kd, 19 kd and 19 kd, respectively. In addition to the PR1 proteins, P and Q are major components of the mixture with apparent mol. wts. of 27 kd and 27.5 kd, respectively. Antisera were raised to a mixture of PR-proteins and to purified 1a, 1b and 1c; these antisera were tested to a number of PR-proteins as shown in Table I. No antibodies to PR2a or 2b were detectable in the antiserum to the mixture of PR-proteins, probably because of the low amount of 2a and 2b in this preparation. A close serological relationship between PR1a, 1b and 1c was found, but the antisera to these proteins did not react with PR2a, 2b, P or Q. When the antisera to PR1a and 1b were intragel adsorbed with PR1b and 1a, respectively, they no longer reacted with the homologous proteins. Our results are in agreement with the observation of Matsuoka and Ohashi (1984) that an antiserum to PR1a of Samsun NN tobacco reacted with PR1b and 1c but not with PR2 and the finding of Antoniw *et al.* (1985) that an antiserum to the b1 protein of Xanthi-nc tobacco reacted with b2 and b3.

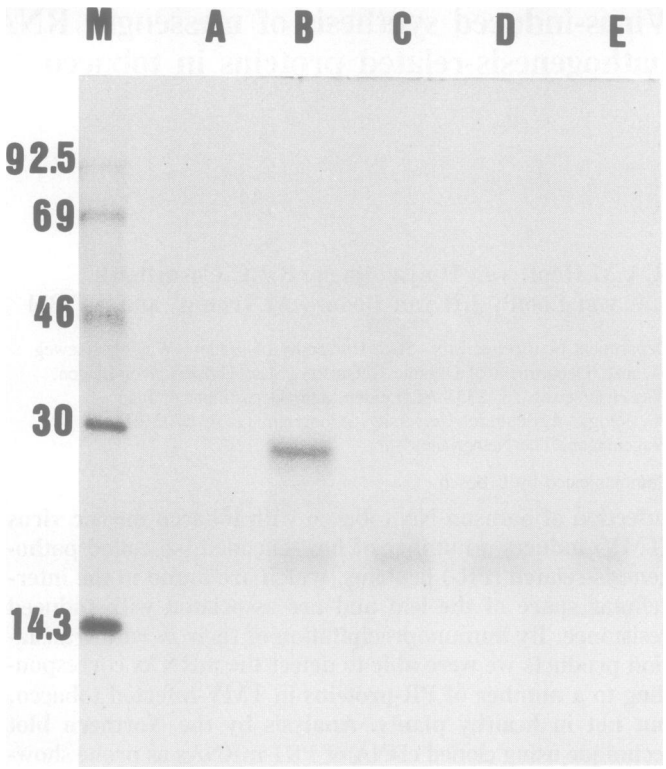

Fig. 3. SDS-PAGE of immunoprecipitated *in vitro* translation products. Poly(A) RNA from TMV-infected tobacco was translated *in vitro* and the radiolabelled products were subjected to immunoprecipitation with pre-immune serum (**lane A**), and antisera raised to unfractionated PR-proteins (**lane B**), PR1a (**lane C**), PR1b (**lane D**) and PR1c (**lane E**). The mol. wt. of marker proteins (**lane M**) is given in kd.

Immunoprecipitation of in vitro synthesized proteins

Poly(A)-containing RNA, isolated from TMV-infected and healthy tobacco plants, was used to direct the *in vitro* synthesis of radiolabelled proteins. PR-proteins present among these products were identified by immunoprecipitation with the four antisera mentioned above. Lanes A and B in Figure 2 show an electrophoretic separation of the proteins translated from RNA isolated from infected and healthy plants, respectively. The RNA from infected plants yields a number of translation products that are precipitable with antiserum directed to unfractionated PR-proteins (Figure 2, lane D). The estimated mol. wts. of the two major bands correspond to those of PR P/Q and 1a. (Individual bands of P and Q could not be resolved in this system.) No such translation products are precipitable from the translation products of RNA from healthy plants (Figure 2, lane F). Figure 2, lanes C and E are the controls with pre-immune serum.

A further analysis of the translation products of RNA from infected plants was done by using the antisera to purified PR1a, 1b and 1c. Figure 3 shows that these three antisera all precipitate the same two bands (lanes C, D and E). Comparison with a lane loaded with unlabeled PR-proteins (not shown) demonstrated that the upper band corresponds to PR1a, whereas the lower band co-migrates with the mixture of PR1b and 1c. No precipitates were obtained when translation products of RNA from healthy

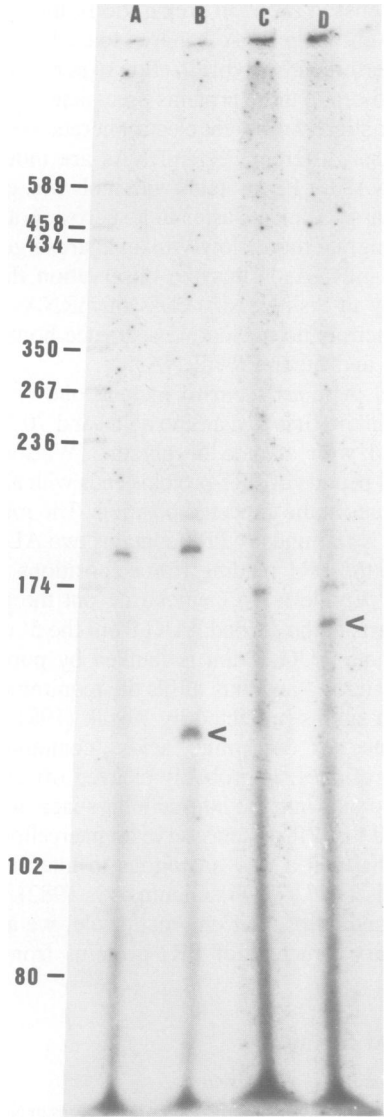

Fig. 4. Autoradiogram of a polyacrylamide gel run with reverse transcription products of poly(A) RNA from healthy (**lanes A** and **C**) and infected (**lanes B** and **D**) tobacco, primed with primer 2 (**lanes A** and **B**) or primer 1 (**lanes C** and **D**). Products specific to PR1-mRNAs are indicated by arrows. Nucleotide numbers of marker DNAs are given at the left.

plants were treated with these antisera (results not shown).

To detect possible size differences between the mRNAs for the respective PR-proteins, poly(A)-containing RNA from infected plants was sedimented in a sucrose gradient. RNA present in the collected fractions of the gradient was translated *in vitro* and the products were analysed by immunoprecipitation using antiserum to the mixture of PR-proteins. The mRNA encoding the P and/or Q proteins (estimated sedimentation coefficient 12S) was found to sediment slightly faster than the mRNA encoding PR1-proteins (estimated sedimentation coefficient 10S) (results not shown).

Leader sequence of PR1-mRNA

Information on the N-terminal amino acid sequence of PR1a (J.Lucas, personal communication) was used to synthesize a deoxyoligonucleotide (primer 1) complementary to the region of the mRNA corresponding to amino acids 9 – 13. This 14-mer was labelled at the 5′ end and used to prime reverse transcription of poly(A) RNA from healthy and TMV-infected tobacco.

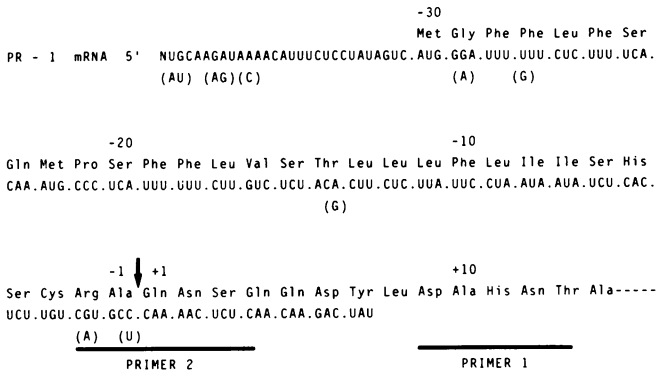

Fig. 5. 5′-Terminal nucleotide sequence of PR1-mRNAs obtained by sequencing the PR1-specific primer extension products shown in Figure 4. The bold arrow indicates the N terminus of PR1a. Deviations from the sequence, found in a minor extension product, are indicated between brackets.

RNA from infected plants yielded two extension products (Figure 4, lane D), one of which was also obtained with RNA from healthy plants (Figure 4, lane C). These bands were eluted from the gel and were partially sequenced. The band unique to RNA from infected plants (arrow in lane D) was found to correspond to PR1-mRNA, the other band is a non-related mRNA, accidently recognized by the primer. Sequence information thus obtained was used to synthesize a second primer (primer 2) hybridizing to PR1-mRNA at a position upstream of primer 1. Again, this primer yielded two major extension products, one of which was unique to RNA from infected plants (Figure 4, lanes A and B). Sequence determination showed that the band indicated with an arrow (Figure 4, lane B) corresponds to PR1-mRNA; the other band is due to priming on a non-related messenger.

Figure 5 shows the leader sequence of PR1-mRNA deduced from the extension products. The bold arrow indicates the N terminus of PR1a (J.Lucas, in preparation). At this position there is no AUG codon in the mRNA. If translation is initiated at the first AUG codon from the 5′ end of the mRNA (nucleotides 30 – 32), PR1a will be synthesized as a precursor with a highly hydrophobic N-terminal extension of 30 amino acids. Probably, this N-terminal sequence functions as a signal peptide.

In fact, sequencing of the primer extension products yielded one major and a closely related minor sequence. The deviations in this minor sequence are indicated between brackets in Figure 5. In some primer extension experiments a minor band migrating somewhat slower than the PR1-specific band in Figure 4, lane B, was obtained. The sequence of this extension product was closely related to but different from the two sequences shown in Figure 5. Thus, evidence was obtained for the existence of three closely related messengers, probably corresponding to PR1a, 1b and 1c.

Induction of PR1-mRNAs

In agreement with the immunoprecipitation data, the results of the primer extension studies indicated that PR-mRNAs are present in infected leaves only. Attempts to confirm these results by hybridizing Northern blots of poly(A) RNA from infected and healthy plants to the labelled primers were unsuccessful, probably because of the low specificity of the primers. Therefore, primer 2 was used as a probe to screen a cDNA library made to poly(A) RNA from TMV-infected tobacco. The construction of this library, the characterization of the clones and their assignment to PR1a, 1b and 1c will be described elsewhere (Cornelissen

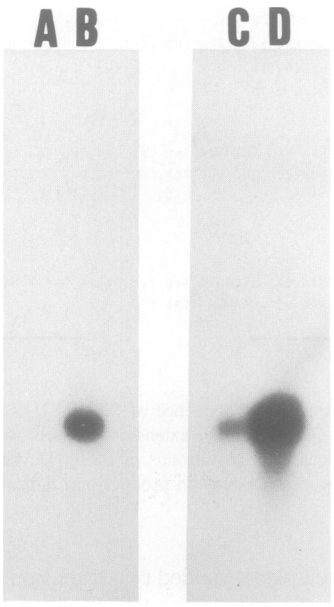

Fig. 6. Induction of PR1-mRNAs by TMV infection of tobacco. Poly(A) RNA from healthy (**lanes A** and **C**) and infected (**lanes B** and **D**) plants was electrophoresed into an agarose gel and blotted to a nitrocellulose filter. The filter was hybridized to a cDNA clone of PR1-mRNA, radiolabelled by nick-translation. The autoradiogram was exposed for 4 h (**lanes A** and **B**) or 46 h (**lanes C** and **D**).

et al., in preparation). Here we show a Northern blot probed with a PR1c clone corresponding to the leader sequence and the sequence coding for the signal peptide and the N-terminal 98 amino acids of the mature PR1c protein. Figure 6 shows the hybridization of this probe to poly(A) RNA from healthy tobacco (lane A) and to poly(A) RNA that was isolated 7 days after inoculation with TMV (lane B). A single band is seen in the lane with RNA from infected plants whereas virtually no signal is obtained with healthy material. However, when the exposure time was increased from 4 h to 46 h (Figure 6, lanes C and D) the autoradiogram showed a weak band of PR1-mRNA in poly(A) RNA from healthy tobacco (lane C). Similar results were obtained when PR1a and 1b clones were used as probe. When tobacco plants were inoculated with buffer, no increase in the level of PR1-mRNAs was observed (results not shown).

Discussion

From their observation that similar amounts of PR1a could be immunoprecipitated from translation products directed *in vitro* by poly(A) RNA from healthy and TMV-infected Xanthi-nc tobacco, Carr *et al.* (1982) concluded that PR-mRNAs are present equally in healthy and infected plants. Our results demonstrate that in Samsun NN tobacco a different situation exists. By immunoprecipitation of *in vitro* translation products, the mRNAs for a number of PR-proteins could be detected in infected plants only (Figure 2). This conclusion was further substantiated for PR1-mRNAs by using cDNA clones as a probe. The Northern blot of Figure 6 shows that low amounts of PR1-mRNA are present in healthy plants but the signal was too weak to allow a reliable comparison with the signal obtained from infected plants. A rough estimate indicates that upon TMV infection the level of PR1-mRNA is increased >100-fold, indicating that synthesis of PR proteins is regulated at the transcriptional level. As the nucleotide sequence homology between PR1-mRNAs is ~90% (Cornelissen *et al.*, in preparation), the relative amounts of PR1a-, 1b- and 1c-mRNA that are induced by TMV infection cannot be determined by hybridization experiments. The observation that antisera to these proteins precipitate *in vitro* synthesized products of slightly different electrophoretic behaviour (Figure 3) indicates that different PR1-mRNAs are induced.

As the mRNAs for PR-proteins with mol. wts. exceeding those of the PR1-class sediment faster in a sucrose gradient, they are expected to migrate more slowly in an agarose gel as compared with the PR1-mRNAs. Thus the observation that PR1-clones hybridize only to a single size class of mRNAs (Figure 6) indicates that there is no nucleotide sequence homology between PR1-mRNAs and other PR-mRNAs.

Most signal peptides seem to include one or more charged residues within the first five amino acids and 70% of the known sequences end with alanine or glycine (Watson, 1984). The putative signal peptide of PR1-proteins ends with alanine but lacks charged residues at the expected position. The mRNA sequence preceding the N terminus of PR1 contains two AUG codons both in-phase with the PR1 reading frame (positions −30 and −22 of the signal peptide). We cannot rule out the possibility that translation starts at the second AUG from the 5′ end. However, the 5′-proximate AUG codon is flanked by purine residues at positions −3 and +4 and thus fulfils the requirements for a functinal initiation site as predicted by Kozak (1981). The putative signal sequence of 30 amino acids, containing >50% of hydrophobic residues, is probably cleaved off during transport of the PR1 proteins into the intercellular space of the leaf. It has been suggested that PR-proteins serve an intercellular 'messenger' function, perhaps in a way analogous to interferons in animal cells (Kassanis *et al.*, 1974; Gianinazzi, 1982). To contribute to an understanding of their enigmatic role, we are now deducing the primary structure of PR1-proteins from the available clones.

Materials and methods

Induction and isolation of PR-proteins

Nine to ten week old tobacco plants (*N. tabacum* cv. Samsun NN) were inoculated with TMV (strain WU1), and PR-proteins were isolated 7 days p.i. as described previously (Antoniw *et al.*, 1980). Individual proteins were purified by chromatofocusing (van Loon, in preparation).

RNA isolation

Leaf material from healthy plants or from plants 7 days after inoculation with 3 μg/ml TMV was harvested, quick-frozen in liquid nitrogen and homogenized in a pre-cooled Waring blender. Two (v/w) volumes of a 85°C phenol buffer solution (50% phenol, 50 mM LiCl, 0.5% SDS, 5 mM EDTA, 50 mM Tris) were added and mixed before addition of a further volume of chloroform. After 30 min shaking at 250 r.p.m. the water phase was again chloroform extracted. The RNA was pelleted in 2 M LiCl and washed twice with 2 M LiCl and 70% alcohol. The RNA was dissolved in a buffer containing 25% formamide, 0.7 M NaCl, 10 mM Hepes, 10 mM EDTA and 0.1% SDS, pH 7.4, heated for 5 min at 65°C, and applied three times on a poly(U)-Sepharose column (Pharmacia). The column was extensively washed with the same buffer containing 50% formamide and 0.5 M NaCl, and the poly(A) RNA was eluted in 95% formamide, 10 mM Hepes, 5 mM EDTA and 0.1% SDS, pH 7.4. The RNA was ethanol-precipitated after addition of 0.25 volumes of 10 M NH_4Ac, washed, and dissolved in sterile H_2O.

Sucrose gradient centrifugation

100 μg poly(A) RNA was layered on a 5−30% linear sucrose gradient in 10 mM Tris-Cl pH 7.8 and 60 mM KCl. After 16.5 h centrifugation at 25 000 r.p.m. in a Beckman SW 50.1 rotor at 4°C, 350 μl fractions were collected, 4.5 μl of which was directly translated in a reticulocyte cell-free system with a final volume of 17.5 μl (see below). A parallel gradient was run with rRNAs as markers.

In vitro translation

Reticulocyte lysate was obtained and used as described by Van Tol and Van Vloten-Doting (1979); incubation mixtures were supplied with 0.3 μCi/ml [^{35}S]methionine

(1460 Ci/mmol, Amersham) and 10 μg/ml poly(A) RNA. After translation, 5% was directly electrophoresed, the rest was used for immunoprecipitation.

SDS-polyacrylamide gel electrophoresis (PAGE)

Protein samples were 1:2 diluted with sample buffer (25 mM Tris, 192 mM glycine, 2.5% SDS, 10% glycerol, 6 M urea, 5% 2-mercaptoethanol pH 8.3), heated for 2 min at 90°C and applied to a 12% polyacrylamide gel (Laemmli, 1970). ^{14}C-Labelled and unlabelled protein mol. wt. markers were purchased from Amersham and Pharmacia, respectively.

Preparation of sera and immunoprecipitation

Rabbit antisera were raised by injecting s.c. and i.m. 1 mg of PR1a, 1b or 1c, or 5 mg of the unfractionated mixture of PR-proteins in complete Freund's adjuvant. After 1 month, 1 mg was re-injected incomplete adjuvant. Serum was collected after 70 days.

To 30 μl translation mixture 'immunebuffer 5×' was added (0.25 M NaH_2PO_4, 0.25 M NaCl, 5% Triton X-100, 2.5% sodium deoxycholate, 0.5% SDS, pH 7.3), 90 μl H_2O and 15 μl pre-immune serum. After 5 min, 20 μl protein A-Sepharose (Pharmacia) was added and the suspension was incubated for 30 min under continuous agitation. The supernatant was recovered and treated with 15 μl of the appropriate serum followed by protein A-Sepharose. The beads were spun through a 0.7 M sucrose cushion and washed twice with immune buffer. All previous steps were performed in ice or at 4°C. The Sepharose slurry was then mixed with sample buffer, heated briefly, and electrophoresed.

Primer extension

The deoxynucleotides 5′ GTGTTGTG(G/T)GCGTC 3′ (primer 1) and 5′ GAGAGTTTTGGGCACG 3′ (primer 2) were synthesized according to Marugg *et al.* (1984). End-labelling was done with 300 ng of the primers, 400 μCi [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase (both NEN). 100 μg poly(A) RNA was dissolved in 10 μl 10 × RT-buffer (0.5 M Tris-Cl, 0.1 M $MgCl_2$, 0.4 M KCl, pH 8.3) and added to the lyophilized primer. The mixture was heated for 1 min at 90°C, cooled gradually to room temperature and adjusted to 100 μl with a final concentration of 0.1 M dithiothreitol, 0.5 mM of dATP, dCTP, dGTP and dTTP each, and 250 U/ml AMV reverse transcriptase (Beard). The transcription proceeded for 30 min at 37°C, and a further 30 min at 42°C. The RNA was OH^--degraded at 60°C and the DNA was precipitated after two phenol extractions. The products were analysed by 7 M urea-5% PAGE (Maniatis *et al.*, 1982). Products were recovered from the gel and the nucleotide sequence determined (Maxam and Gilbert, 1980).

Northern blots

Poly(A) RNA (20 μg per slot) was electrophoresed in agarose gels, blotted to nitrocellulose filters and hybridized to radiolabelled probes by the procedures described by Sarachu *et al.* (1985).

Acknowledgements

We are grateful to Drs. J.Lucas and A.Henschen (Protein Chemistry Department II, Max-Planck Institute of Biochemistry, 8033 Martinsried bei München) for sending us a manuscript prior to publication. This work was sponsored in part by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

References

Antoniw,J.F .and White,R.F. (1980) *Phytopathol. Z.*, **98**, 331-341.
Antoniw,J.F., Ritter,C.E., Pierpoint,W.S. and Van Loon,L.C. (1980) *J. Gen. Virol.*, **47**, 79-87.
Antoniw,J.F., White,R.F., Barbara,D.J., Jones,P. and Longley,A. (1985) *Plant Mol. Biol.*, **4**, 55-60.
Carr,J.P. (1983) *Neth. J. Plant Pathol.*, **89**, 311-312.
Carr,J.P., Antoniw,J.F., White,R.F. and Wilson,T.M.A. (1982) *Biochem. Soc. Trans.*, **10**, 353-354.
Fraser,R.S.S. (1982) *J. Gen. Virol.*, **58**, 305-313.
Gianinazzi,S. (1982) in Wood,R.K.S. (ed.), *Active Defense Mechanisms in Plants*, Plenum Press, NY, pp. 275-298.
Gianinazzi,S., Pratt,M.H., Shewry,P.R. and Miflin,B.J. (1977) *J. Gen. Virol.*, **34**, 345-351.
Kassanis,B., Gianinazzi,S. and White,R.F. (1974) *J. Gen. Virol.*, **23**, 11-16.
Kozak,M. (1981) *Nucleic Acids Res.*, **9**, 5233-5252.
Laemmli,U.K. (1970) *Nature*, **227**, 680-685.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) in *Molecular Cloning: A Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.
Marugg,J.E., Piel,N., McLaughlin,L.W., Tromp,M., Veeneman,G.A. and Van Boom,J.H. (1984) *Nucleic Acids Res.*, **12**, 8639-8651.
Matsuoka,M. and Ohashi,Y. (1984) *J. Gen. Virol.*, **65**, 2209-2215.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499-560.
Parent,J.-G. and Asselin,A. (1984) *Can. J. Bot.*, **62**, 564-569.
Pierpoint,W.S. (1983) *Phytochemistry*, **12**, 2691-2697.
Sarachu,A.N., Huisman,M.J,. Van Vloten-Doting,L. and Bol,J.F. (1985) *Virology*, **141**, 14-22.
van Loon,L.C. (1975) *Virology*, **67**, 566-575.
van Loon,L.C. (1982) in Wood,R.K.S. (ed.), *Active Defense Mechanisms in Plants*, Plenum Press, NY, pp. 247-273.
van Loon,L.C. (1985) *Plant Mol. Biol.*, **4**, 111-116.
Van Tol,R.G.L. and Van Vloten-Doting,L. (1979) *Eur. J. Biochem.*, **93**, 461-468.
Wagih,E.E. and Coutts,R.H.A. (1982) *Phytopathol. Z.*, **104**, 364-374.
Watson,M.E.M. (1984) *Nucleic Acids Res.*, **12**, 5145-5164.

Received on 28 May 1985; revised on 27 June 1985