Plant Molecular Biology **30**: 493–504, 1996.

The *PR-1a* promoter contains a number of elements that bind GT-1-like nuclear factors with different affinity

Annemarie S. Buchel, Richard Molenkamp, John F. Bol and Huub J.M. Linthorst*
*Institute of Molecular Plant Sciences, Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA Leiden, Netherlands (*author for correspondence)*

Received 19 June 1995; accepted in revised form 6 November 1995

Key words: GT-1, induced expression, PR proteins, salicylic acid, *trans*-acting factors

Abstract

The 900 bp promoter region of the tobacco *PR-1a* gene was divided into eight fragments using PCR. The fragments were tested for their ability to bind to nuclear factors isolated from tobacco leaf. Band shift assays demonstrated that all but one of the fragments specifically interacted with nuclear proteins. From competition experiments it was determined that the same nuclear factors bind various promoter fragments with different affinity. Moreover, efficient competition with a synthetic tetramer of box II of the *rbcS* promoter (Green PJ *et al.*, EMBO J 13 (1988) 4035–4044) indicated that GT-1-like nuclear factors are involved in these interactions. Furthermore, in comparison to extracts from untreated plants, nuclear protein preparations from tobacco mosaic virus-infected tobacco showed a reduced GT-1 binding activity. These results will be discussed in relation to induced *PR-1a* gene expression.

Introduction

Plant pathogenesis-related (PR) proteins accumulate upon pathogen attack and other stress conditions. Five groups of tobacco PR proteins (PR-1 to PR-5), induced during the hypersensitive response to tobacco mosaic virus (TMV) infection, can be divided into sets of acidic, extracellular and basic, vacuolar counterparts. The genes for different proteins in each set are coordinately induced by various stress conditions and respond to treatment of the plant with hormones such as salicylic acid (SA) and ethylene [5]. They include genes encoding PR-1 proteins, β-1,3-glucanases (PR-2), chitinases (PR-3), and osmotin-like proteins (PR-5), which have been implicated to be involved in acquired resistance to fungal infection [2, 36, for reviews see 4, 19].

Tobacco contains three active genes encoding extracellular PR-1 proteins [6, 23, 24, 26, 27]. The nucleotide sequences of the *PR-1* genes and their flanking regions are highly similar. Previous studies from our group with transgenic plants containing *PR-1a* upstream sequences fused to the β-glucuronidase (GUS) reporter gene have indicated that for efficient, high level expression at least 0.9 kb of upstream promoter sequence is necessary. Deleting the promoter to bp −689 resulted in decreased but still inducible expression, whereas a further deletion to −643 destroyed SA and TMV inducibility [33]. Similar results were obtained by Ukness *et al.* [30], who found a 661 bp *PR-1a* promoter capable of inducible GUS gene expression. Upstream sequences of over 600 bp have also been found to be required for induction of the genes encoding acidic and

basic PR-2 proteins and acidic PR-5 proteins [32, 1].

A more detailed analysis of the *PR-1a* promoter resulted in the identification of a minimum of four regulatory elements, located at nucleotides −902/−691, −689/−643, −643/−287 and −287/+29. Alone, these elements were found to have no promoter activity and all four were required for maximum induction of the GUS reporter gene by treatment of plants with SA or infection with TMV. Constructs containing two or three of these elements had about half of this maximum activity [31]. In contrast to the results of van de Rhee and Bol [31], Uknes *et al.* [30] and Beilmann *et al.* [3], who found no significant induction with the −287/+29 bp fragment of the *PR-1a* promoter, Ohshima *et al.* [25] reported that this fragment was sufficient for SA- and TMV-inducible expression. In an interspecific *Nicotiana* hybrid, constitutively expressing the *PR-1a* gene, this 300 bp promoter region lacked specific interaction with a DNA-binding protein which was present in tobacco [13]. The binding of this protein factor was suggested to repress *PR-1a* gene expression in non-induced tobacco.

To investigate the binding of possible regulatory proteins to the 906 bp 5′-flanking *PR-1a* sequence in induced and non-induced tobacco, we divided this region into eight fragments by PCR. Nuclear proteins were extracted from non-induced, SA-treated and TMV-infected Samsun NN tobacco plants. Gel retardation assays revealed that all but one of the eight promoter fragments specifically interacted with nuclear proteins. Competition experiments indicated that these fragments did bind to similar GT-1-like proteins with different affinities.

Materials and methods

DNA fragments box II, AS-1, competitor DNA

The tetramers of box II with core sequence GGTTAA, and box II mutants, with GG replaced by CC (mutant 1) and TT replaced by GG (mutant 2 [12]) and of AS-1 [17] were obtained from their plasmid vectors by restriction enzyme digestion, and after electrophoresis were eluted from agarose gel. As a non-specific competitor an internal 231 bp fragment of the β-glucuronidase (GUS) gene was derived from pBI101 [14] after digestion with *Eco*RV.

Preparation of PR-1a *promoter fragments*

The region of the *PR-1a* gene from −906 bp to +29 bp, relative to the transcription start site, was divided in fragments A to H by PCR. Most PCR primers contained additional 5′ nucleotides providing restriction sites for cloning. The sequence and position relative to the transcription start site of the various primers is listed in Table 1. The numbering of nucleotides in the *PR-1a* upstream sequence is according to Payne *et al.* [26]. DNA amplification was performed using DNA polymerase from *Thermus aquaticus* (BRL) to allow relatively low annealing temperatures. To enable a more efficient ligation and restriction, the resulting DNA fragments were used as templates in a second round of amplification by PCR using *Thermococcus litoralis* DNA polymerase (Vent Polymerase, Biolabs). The PCR products were gel-purified and cloned and the identity of each cloned fragment was confirmed by sequencing.

Gel retardation and filter binding assays

Nuclear protein extracts were prepared as described by Green *et al.* [10] from leaves of non-induced tobacco (*Nicotiana tabacum* cv. Samsun NN), from induced tobacco leaves after four daily sprayings with 10 mM Na-SA, or four days after inoculation with TMV. DNA fragments for gel retardation assays obtained after digestion with the proper restriction enzymes, were gel-purified and end-labelled with α-^{32}P-dCTP, using Klenow polymerase. DNA-binding reactions typically contained 0.5 ng labelled DNA fragment, 3 μg poly(dIdC)·(dIdC) (Pharmacia, Uppsala, Sweden), and 5 μg nuclear protein extract, in 10 μl of binding buffer (20 mM HEPES pH 6.7, 40 mM

Table 1. Primers used for amplification of *PR-1a* promoter fragments.

Primer[1]	Sequence[2]	Position[1]
A1	*XhoI* **CACCCTCGAGGATTTCAAAC**	-906
A2	*Bam* HI **CGCGGATCCCAATCACGACTTGAC**	-764
B1	*Bam* HI **GCGGGATCCTAAAGTCAAGTCGTG**	-787
B2	*Bam* HI **CGCGGATCCTTCCCTTAATTTCC**	-656
C1	*Bam* HI **GCGGGATCCGGAAATTAAGGGAAGG**	-672
C2	*Nco* I **CGCCCATGGCCGTCATCTCGATG**	-571
D1	*Bam* HI **GCGGGATCCAAATATTCTTTACGTCC**	-632
D2	*Eco* RI **CGCGAATTCTTATATGTACAATCAATTCT**	-467
E1	*Xho* I **GCGCTCGAGAGAATTGATTGTAC**	-492
E2	*Pst* I **CGCCTGCAGAATGGGAATGTCC**	-375
F1	*Nco* I **GCGCCATGGACATTCCCA**	-392
F2	*Bgl* II **CGCAGATCTATCAGATGTGC**	-253
G1	*Bam* HI **GCGGGATCCGCACATCTGATAGATC**	-268
G2	*Bam* HI **CGCGGATCCTATTTTCTAGGTTACC**	-131
H1	*Nco* I **GCGCCATGGTAACCTAGAAAATAGG**	-157
H2	*Nco* I **CGCCCATGGAGAAATGTTGTATC**	+22

[1] Upstream (1) and downstream (2) primers are named according to the corresponding fragments (see Fig. 1).

[2] The region in bold indicates identity to the sequence of the *PR-1a* gene. Restriction sites are indicated.

[3] The number indicates the first (for upstream primers) or the last (for downstream primers) nucleotide identical to the sequence of the *PR-1a* gene, relative to the transcription start site.

KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.8 mM PMSF). Additional specific or non-specific competitors were added as indicated. After binding for 30 min at room temperature the samples were electrophoresed at 100 V in a 5% (w/v) polyacrylamide gel in 44.5 mM Tris-borate, 44.5 mM boric acid, 1 mM EDTA. The gel was subsequently dried onto chromatography paper and exposed to X-ray film at −80 °C. The relative amount of retarded DNA was measured using a blot analyzer (Betascope 603, Betagen). Filter binding assays with plaque lifts of λ phage expressing the GT-1-coding sequence were performed in the above binding buffer, essentially as described by Gilmartin *et al.* [9].

Results

Interaction of nuclear proteins with PR-1a promoter fragments

To analyze the binding of tobacco nuclear proteins to the *PR-1a* promoter, gel retardation experiments were performed. Previously, the upstream region of 906 bp of the *PR-1a* promoter was found to be sufficient for 60-fold induction of gene expression [31, 33]. In the current experiments this promoter region was divided into eight similarly sized fragments by PCR. A schematic representation of the promoter fragments A to H is shown in Fig. 1. Each fragment was tested for its ability to specifically interact with proteins from a tobacco nuclear extract. Figure 2 shows the results of a representative band-shift assay with fragment A. Upon incubation with the nuclear extract, part of the labelled fragment A was retarded in three more slowly migrating bands (lane P). The nature of the fast migrating band, immediately above the position of the unbound probe was not further investigated.

Addition to the binding assay mixture of increasing amounts of unlabelled fragment A as specific competitor (lanes S) showed that a 10-fold molar excess efficiently inhibited the formation of the two slowly migrating labelled complexes. The same molar excess of non-specific GUS-competitor DNA had no effect. In fact,

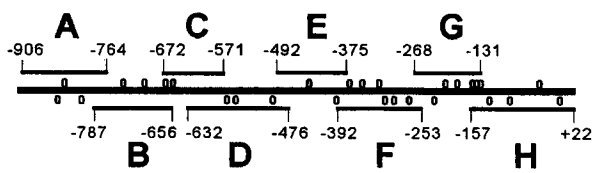

Fig. 1. Fragments of the *PR-1a* promoter. Schematic representation of the *PR-1a* promoter region from −906 to +29. The position of the ends of fragments A to H, relative to the *PR-1a* promoter (solid line), is indicated. Putative GT-1 binding sites present in the different fragments are indicated (°).

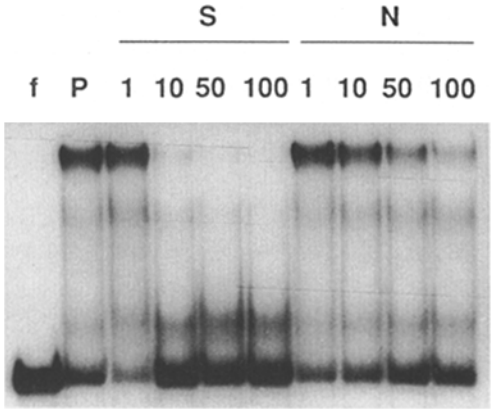

Fig. 2. Specific interaction of nuclear proteins with *PR-1a* promoter fragment A. A nuclear protein extract from non-induced tobacco was incubated with radioactively labelled *PR-1a* promoter fragment A, without (lane P) or with competitor DNA. In lanes S increasing amounts of unlabelled fragment A was added as specific competitor at molar excesses of 1-, 10-, 50-, or 100-fold, as indicated. Lanes N contain a similar series of increasing amounts of non-specific competitor (a fragment of the coding region of the GUS gene). Lane f contains probe without addition of nuclear proteins. The samples were electrophoresed in polyacrylamide gels after which the gels were autoradiographed.

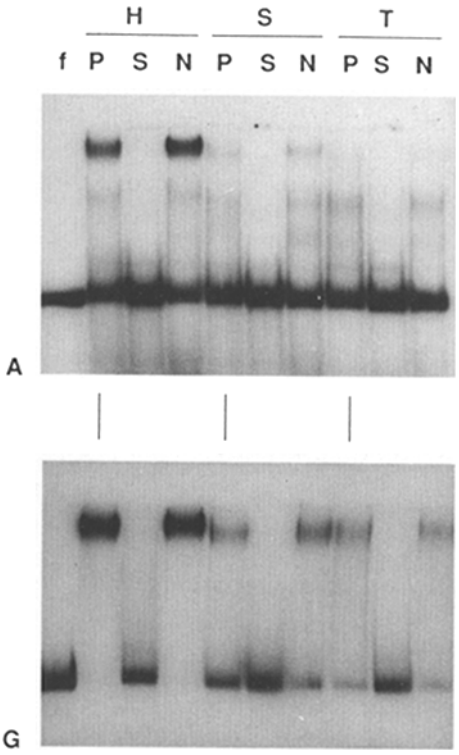

Fig. 3. Interaction of nuclear proteins with *PR-1a* promoter fragments. Nuclear protein extracts from non-induced (H), SA-treated (S), and TMV-infected (T) tobacco leaf were incubated with end-labelled DNA in the absence (lanes P) or presence of a 100-fold molar excess of the same unlabelled DNA as specific competitor (lanes S) or a 100-fold molar excess of a fragment from the GUS coding region as non-specific competitor (lanes N). Lanes f contain the DNA probe without added nuclear extract. In the upper panel *PR-1a* promoter fragment A was used to a probe and in the lower panel fragment G.

even a 100-fold molar excess of GUS DNA was not able to completely compete for binding (lanes N). To determine if the retarded bands found in this assay represent DNA-protein interactions, the extract was treated to denature the proteins. Heating of the nuclear protein extract for 10 min at 65 °C or treatment with 4 μg proteinase K for 10 min at 22 °C prior to incubation with the fragment completely abolished the appearance of retarded bands, while treatment with 4 μg RNase A for 10 min at 22 °C had no effects (results not shown).

Under the conditions used, also fragments B, D, E, F, G and H were capable of interacting with the tobacco nuclear proteins (results not shown). Apart from fragment C, all fragments specifically complexed with proteins, resulting in band-shifts with similar mobility as the slower migrating, fragment A-containing complex (see also Fig. 3, lower panel). The complex formed with fragment C appeared not to be specific, since the interaction was inhibited in the presence of low amounts of the non-specific GUS competitor (results not shown).

Complex formation with different nuclear extracts

Figure 3 shows the results of band-shift assays with the nuclear extracts from non-induced, SA-induced and TMV-infected tobacco. In the upper panel, fragment A was used to a probe for complex formation, again showing that binding to nuclear proteins from non-induced tobacco (H) resulted in three retarded bands. The same com-

plexes were formed when the binding assays were done with the nuclear extract from SA-induced tobacco (S). However, the amount of the slower migrating complex was greatly reduced. This effect was even more evident with the nuclear extract from TMV-infected tobacco (T), where there was an almost complete absence of this complex formation.

The same technique was used to determine whether the other fragments complexed with proteins from the three nuclear extracts with different efficiency. The lower panel of Fig. 3 shows that binding of fragment G resulted in a similar specific, slowly migrating complex. Although less obvious, also here, incubation with the nuclear proteins from induced leaves resulted in lower levels of binding. The graphical representation of complex formation shown in Fig. 4 illustrates that, under the conditions used (5 μg of nuclear protein and 0.5 ng labelled probe in 10 μl), also fragments B and D gave rise to elevated binding with the nuclear proteins from non-induced tobacco. This effect was most evident with the far upstream fragments A and B, where binding to the nuclear proteins of TMV-infected tobacco was reduced to 10–20% of the binding with the non-induced extract. The differences in binding with fragments F and H were less significant. The higher binding with the extract from SA-treated tobacco to fragment E was not substantiated by the binding to the TMV-infected extract. The complex formed with fragment C appeared to be not specific, since it was efficiently competed by the non-specific competitor.

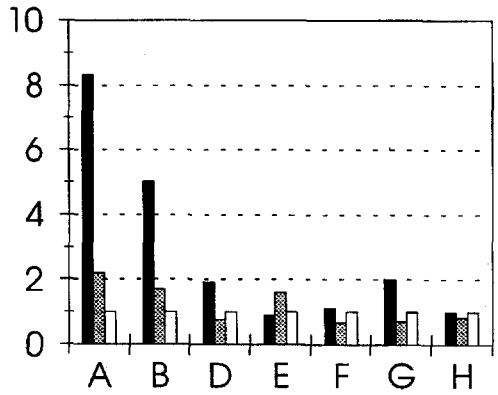

Fig. 4. Relative binding of nuclear proteins from non-induced and induced extract to *PR-1a* promoter fragments. The amount of radioactively labelled complex formed upon incubation of 0.5 ng of the indicated *PR-1a* promoter fragment with 5 μg of nuclear proteins isolated from non-induced tobacco (black bars) and SA-treated plants (grey bars) was quantitated and displayed relative to the amount of complex formed with the same amount of nuclear protein extract from TMV-infected plants (white bars). Since no specific complex formation was detected with fragment C it is not included.

Competition between different PR-1a promoter fragments

Upon incubation with tobacco nuclear extracts, most promoter fragments used in the above band-shift experiments appeared to be retarded in complexes with low mobility. To investigate whether the same nuclear protein(s) are involved in these shifts, competition experiments were performed, in which binding to one labelled promoter fragment was competed for by addition of the other unlabelled promoter fragments. The results of two representative experiments are shown in Fig. 5. The upper panel of Fig. 5 shows that formation of the large complex formed with labelled fragment A and nuclear proteins from non-induced tobacco (lane P) was efficiently inhibited by addition of a 10-fold molar excess of unlabelled fragment A. Also, addition of a 10-fold molar excess of fragments B, D, F, G and H inhibited formation of this complex, while fragments C and E did not compete for binding. In the case of fragment C, this could be explained by the fragment's inability to specifically interact with proteins of the tobacco nuclear extract.

The lower panel of Fig. 5 displays the results of a similar competition experiment with fragment G as labelled probe. It is evident that in this case, apart from unlabelled fragment G, only fragments F and H efficiently competed for complex formation. Table 2 contains the combined results of the competition experiments with all *PR-1a* promoter fragments. The table shows that fragments F, G and H were capable of competing for protein binding with any of the *PR-1a* promoter fragments, whereas the more upstream fragments A,

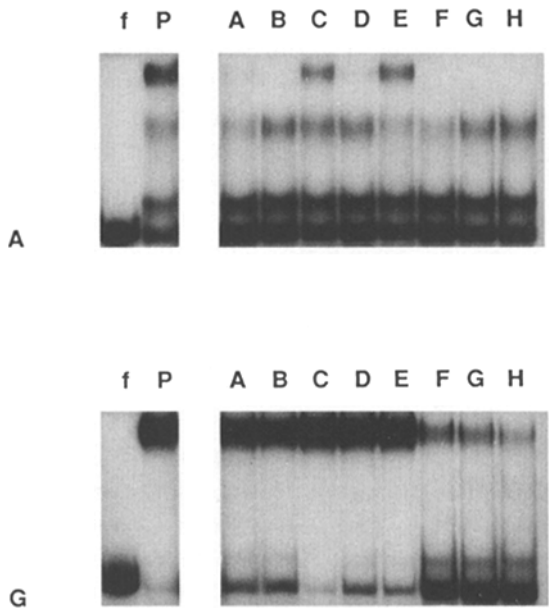

Fig. 5. Competition between *PR-1a* promoter fragments for nuclear factors. Band shift experiments showing complex formation with nuclear proteins from non-induced tobacco and *PR-1a* promoter fragments A (upper panel) and G (lower panel) in the absence (lanes P) or presence of a 10-fold molar excess of non-labelled competitor *PR-1a* promoter fragment A to H, as indicated. Lanes f contain the probe in the absence of nuclear extract.

Table 2. Competition of complex formation with tobacco nuclear proteins between different fragments of the *PR-1a* promoter.

Probe	Competitor[1]							
	A	B	C	D	E	F	G	H
A	+ +	+ + +	–	+ +	–	+ + +	+ + +	+ + +
B	+ +	+ +	–	+ +	–	+ + +	+ + +	+ + +
C	+ +	+ +	+	+ +	+ +	+ + +	+ + +	+ + +
D	+ +	+ +	–	+ + +	+ +	+ + +	+ + +	+ + +
E	+ +	+ +	–	+ +	+	+ + +	+ + +	+ + +
F	–	–	–	–	–	+ +	+ + +	+ + +
G	–	–	–	–	–	+ + +	+ + +	+ + +
H	–	–	–	–	–	+ +	+ + +	+ + +

[1] A 10-fold molar excess of competitor DNA over labelled probe was used in a binding assay with nuclear protein extracted from non-induced tobacco. The number of plus signs indicates the relative amount of competition in formation of the slowly migrating complex, as determined from gel electrophoretic analysis. Minus signs indicate the absence of competition.

B and D did not compete with fragments F, G and H. Fragment E efficiently competed only with fragments C and D. Fragment C did not compete with any of the other fragments. This is in line with the finding that also the non-specific GUS DNA competed for binding to fragment C.

These data support the assumption that the same protein(s) bind to be different *PR-1a* promoter fragments. Furthermore, they indicate that the binding to the far upstream fragments (A, B, D, E) is weaker than the binding to the fragments closer to the transcription start site (fragments F, G, H).

Binding of GT-1 like proteins to the PR-1a promoter

For the routine analysis of the quality of the different nuclear extracts two probes for well-characterized nuclear factors were used. These were tetrameric oligonucleotides of the AS-1 enhancer of the cauliflower mosaic virus (CaMV) 35S promoter [17] and the box II element in the promoter of the gene for the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS-3A*) of pea [11]. The AS-1 enhancer interacts with the plant leucine zipper protein ASF-1 [17]. This binding is crucial for the auxin responsiveness of the 35S RNA expression [20]. The upper panel of Fig. 6 shows the results of binding of tobacco nuclear proteins to the AS-1 tetramers. It is evident that the extracts from non-induced (H), SA-treated (S) and TMV-infected (T) tobacco contain factors interacting with the probe, resulting in two band shifts (lanes P). The specificity of the interaction between the AS-1 DNA and the tobacco nuclear proteins was checked in competition assays with specific and non-specific competitors. Addition of a 50-fold excess of unlabelled AS-1 DNA to the binding assay resulted in a reduction in the amount of shifted probe (lanes S), whereas a 50-fold excess of a similarly sized fragment of the GUS coding region as a non-homologous competitor did not result in decreasing quantities of shifted probe (lanes N). The higher intensity in the three lanes containing the complexes formed with the nuclear extract from

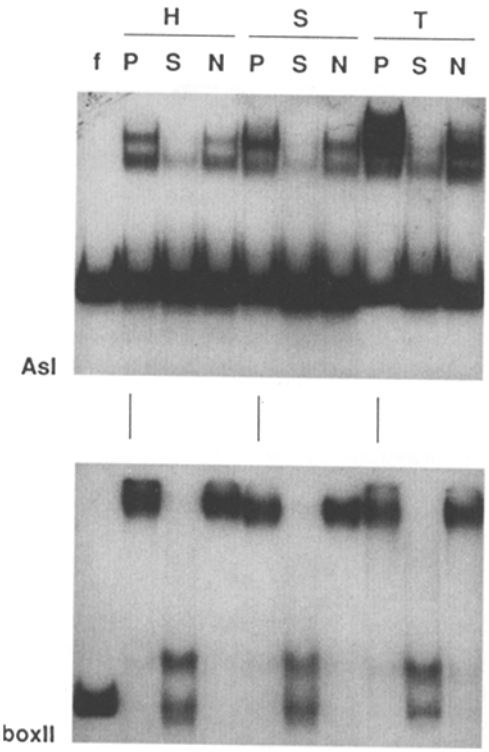

Fig. 6. Interaction of nuclear proteins with fragments AS-1 and box II. In the upper panel the probe was a tetramer of the AS-1 enhancer of the CaMV 35S promoter. The probe used in the lower panel is a tetramer of element II of the *rbcS-3A* gene (see text). For details see legend to Fig. 3.

infected tobacco indicates that TMV-infection results in an increase of ASF-1 binding activity (lanes T).

Box II and related motifs (box II*, box II**, box III, boxIII*, box III**) are conserved elements in the pea *rbcS-3A* gene necessary for light-responsive expression. They specifically bind nuclear protein factor GT-1 [12]. With box II DNA as probe in our band-shift experiments similar results were obtained as with AS-1, although there was no increased binding with the induced extracts. The bottom panel of Fig. 6 shows that binding of tobacco GT-1-like factors resulted in the formation of a slowly migrating complex which could only be competed for by unlabelled box II DNA. GT-1 binding appears to be very efficient since the same amount of extract as was used in the other band-shift experiments (5 μg of protein per binding assay) resulted in complex formation of all 0.5 ng of box II probe. At lower protein to probe ratios differences in GT-1 binding efficiency between nuclear protein extracts from non-induced and TMV-infected tobacco became evident. The results presented in Fig. 7 indicate that extracts from non-infected plants contain approximately two-fold higher GT-1 binding activity to the box II probe at nuclear extract concentrations below 0.3 mg/ml.

The similar mobility of the shifts produced by box II and the various *PR-1a* promoter fragments prompted us to investigate if GT-1-like proteins were involved in the binding to the *PR-1a* promoter fragments. Therefore, competition experiments were carried out in which complex formation with AS-1 and box II DNA were studied in the presence of promoter fragments A and G. As can be seen in the top left panel of Fig. 8, a 50-fold molar excess of fragments A and G did not compete for the binding of ASF-1 to AS-1 DNA. However, in the top right panel of Fig. 8 addition of a 50-fold excess of fragment A resulted in a small reduction of complex formation of GT-1 to box II DNA, whereas an excess of fragment G

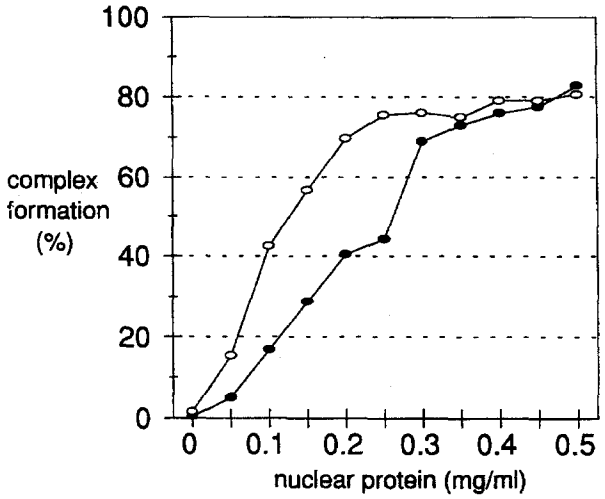

Fig. 7. Titration curves of box II binding activity in tobacco nuclear extracts. The amount of shifted box II probe, measured by band shift assay at different nuclear protein concentrations, is indicated as a percentage of the total amount of probe. Nuclear extracts were derived from non-induced tobacco (open symbols) and TMV-infected tobacco (closed symbols).

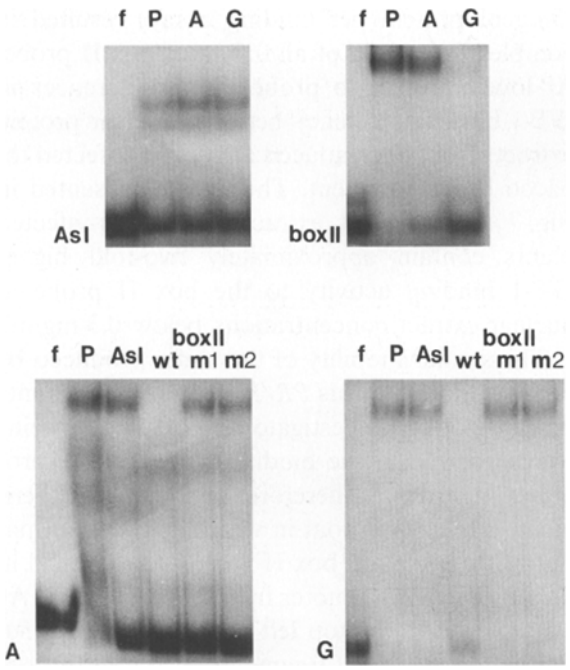

Fig. 8. Binding of GT-1-like proteins to *PR-1a* promoter fragments A and G. Band shift experiments with nuclear proteins from non-induced tobacco with as probes AS-1 DNA (top left panel), box II DNA (top right panel), *PR-1a* promoter fragment A (bottom left panel) and *PR-1a* promoter fragment G (bottom right panel). Lanes f contain the probe without addition of nuclear extract and lanes P contain the complexes formed without the addition of unlabelled competitor DNA. The other lanes contain the complexes formed in the presence of a 50-fold molar excess of unlabelled competitor DNA *PR-1a* fragment A (A), *PR-1a* fragment G (G), AS-1 (AsI), box II (boxII, wt), box II mutant 1 (boxII, m1) and box II mutant 2 (boxII, m2).

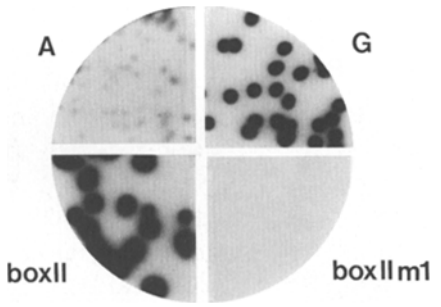

Fig. 9. Plaque lifts containing GT-1 protein expressed from phage, bound to different probes. A nitrocellulose filter disc with GT-1 expressed in *E. coli* infected with lambda phage containing the GT-1 coding sequence, was devided in four parts. Each part was separately incubated with a radioactively labeled probe (A and G, *PR-1a* fragments A and G, respectively; boxII and boxIIm1, box II and mutant box II DNA, respectively).

almost completely blocked GT-1 binding to box II. This difference in competition efficiency between fragments A and G may be due to a lower affinity of fragment A for GT-1 and is reflected by the complementary competition experiments between fragments A and G shown in Fig. 5 and Table 2.

Further evidence for the involvement of GT-1 in *PR-1a* promoter binding comes from the reverse experiment in which complex formation of probes A and G was studied in the presence of excess AS-1 or box II DNA. The bottom left panel of Fig. 8 shows that AS-1 could not interfere with the binding to fragment A (lane AS1), whereas box II completely blocked binding (lane boxII, wt). However, when box II mutants were used as competitor, which were unable to bind GT-1 *in vitro* [12], binding to fragment A was not disturbed (lanes boxII, m1 and m2). The same result was obtained with *PR-1a* promoter fragment G as probe (Fig. 8, bottom right panel) and with fragment H (results not shown). With both fragments G and H complex formation was highly efficient under our standard conditions (5 μg nuclear protein extract per 0.5 ng probe), as can be seen from the high proportion of shifted probe. Finally, in Fig. 9 it is shown that plaque lifts from lambda phage-infected *E. coli* expressing GT-1 [9] bind PR-1a promoter fragments A and G and the tetrameric box II DNA, whereas the box II mutant 1 probe does not bind.

Discussion

Our earlier studies [31, 33] indicated that induction of the *PR-1a* gene by SA treatment or TMV infection required an interaction between multiple regulatory elements that are distributed over a sequence of 900 bp upstream of the gene. These elements were assigned to four fragments ($-902/-689$, $-689/-643$, $-643/-287$ and $-287/+29$) of this promoter region which were all nec-

essary for maximum inducibility of the GUS reporter gene. The present work shows that these fragments contain one or more binding sites for nuclear proteins (probably GT-1). Binding sites in fragment −902/−643 are reflected by the specific interactions of fragments A and B with nuclear proteins. Fragment −689/−643 overlaps 33 nucleotides with fragment B and 29 nucleotides with fragment C. Fragment C did not bind specifically to nuclear proteins at all. Possibly, *cis*-acting elements of the −689/−643 sequence are incompletely represented in fragments B and C. The −625/−287 fragment overlaps the sequences of fragments D, E and F, which all specifically bind nuclear proteins. The observation that fragment C shows no specific binding suggests that specific binding sites in fragment D are located in the region −571/−476. Finally, fragment −287/+29 overlaps with fragments F, G and H which all contain specific binding sites for nuclear proteins.

The data from the competition experiments summarized in Table 2 indicate that a single type of protein is binding to promoter fragments A, B, D, E, F, G and H. At a 10-fold excess the unlabelled fragments F, G and H compete with the binding of protein to labelled fragments A, B, D and E even more efficiently than the homologous competitors do, suggesting that the binding sites in fragments F, G and H have a relatively high affinity for the nuclear protein. This difference in affinity is confirmed by the observation that at a 10-fold excess the unlabelled fragments A, B, D and E do not compete with the binding of the nuclear protein to labelled fragments F, G and H. Among fragments A, B, D and E, fragment E seems to have the lowest affinity for the nuclear factor as fragments A, B and D compete more efficiently with the labelled fragment E than the homologous fragment does. While the findings presented in Fig. 5 and Table 2 demonstrate that the same nuclear protein(s) interact with the different *PR-1a* promoter fragments, the experiments with box II DNA shown in Figs. 8 and 9 indicate that GT-1 like factors specifically interact with *PR-1a* promoter fragments A and G. Together these data indicate that the *PR-1a* promoter contains a number of GT-1 binding sites distributed over the entire 900 bp upstream region.

The nuclear factor GT-1 is known to bind to several degenerated DNA motifs present in the promoter of the pea *rbcS-3A* gene [11, 12,

Table 3. Putative GT-1 elements present in the *PR-1a* promoter.

Fragment[1]	Position[2]	Sequence[3]	
GT-1 core consensus[1]		G G A A A A A T T T C	
A	−854	G G A A A A	(rev)
	−844	G A A A A A	
	−816	G T A A A T	(rev)
B	−761	G A A A A T	
	−713	G A T A A T	
B/C	−672	G G A A A T	
C	−658	G G A A A T	
C/D	−592	G T T A A A	(rev)
D	−559	G G A A A A	(rev)
	−497	G A A A A A	(rev)
E	−435	G A A A A T	
	−394	G G A A A A	(rev)
F	−375	G A A A A A	
	−349	G T A A A A	
	−321	G A A A A T	
	−306	G T T A A T	(rev)
	−297	G G T T A A	(rev)
	−274	G T T A A A	(rev)
G	−238	G T T A A A	(rev)
	−199	G G T A A A	
	−176	G G T A A C	
G/H	−150	G G T A A C	
	−141	G A A A A T	
H	−133	G A T A A A	
	−123	G A T A A T	(rev)
	−93	G G T A A T	(rev)
	−17	G T A A A T	
	−1	G G A A A A	(rev)

[1] The consensus sequence of the GT-1 binding sites according to Green *et al.* [12] is given.

[2] The position of the first G-residue of the putative GT-1 binding sites in the *PR-1a* promoter is given.

[3] The sequences labelled (rev) are from the bottom strand. All sequences are from 5′ to 3′.

Table 3]. Similar sequence motifs have been identified in other light-responsive genes (oxygen evolving complex component of potato [29], pea ferredoxin [8], rice phytochrome [15], *Nicotiana plumbaginifolia* chlorophyll *a/b*-binding protein [28]) and in other inducible plant genes like the defense gene encoding chalcone synthase of bean [18] and the anaerobic response gene of maize encoding maize alcohol dehydrogenase [16]. These data suggest that GT-1 is a general transcription factor recognizing conserved binding sites in various inducible genes of different plants species.

By comparison of the six GT-1-binding sites in the promoter of the *rbcS-3A* gene the consensus sequence G-Pu-(T/A)-A-A-(T/A) was identified [12]. Based on this sequence, a number of potential GT-1-binding sites can be distinguished in the *PR-1a* promoter. Table 3 shows a comparison of these putative binding sites to the GT-1 consensus sequence, while the position of these sites in the *PR-1a* promoter is visualized in Fig. 1. It has been shown that *rbcS-3A* box II (GGTTAA) has a higher affinity for GT-1 than box III (GAAAAT) [9]. *PR-1a* promoter fragment F contains an exact copy of the critical core sequence of *rbcS-3A* box II. Sequences identical to the *rbcS-3A* box III core are found in fragments B, E, F, G and H, whereas the homologous box II* (GGTAAT) and box II** (GGTAAC) [12] are present in fragments G and H. The differences in ability of the *PR-1a* promoter fragments to compete for protein binding to box II (Fig. 8) and to the other promoter fragments (Fig. 5) could be explained by the presence of motifs with different affinity for GT-1-like proteins.

After TMV infection or SA treatment the binding of the nuclear protein to most *PR-1a* promoter fragments was considerably reduced. In the course of these experiments six batches of nuclear proteins were isolated in parallel from non-induced, SA-induced and TMV-infected plants. At equal total protein concentration the extracts from non-induced and TMV-infected tobacco showed a reproducible 2- to 3-fold difference in GT-1-binding activity to box II DNA (see also Fig. 7). Titration experiments with fragment A demonstrated that at certain protein/probe ratios an up to 10-fold higher binding activity was present in the extracts from the non-induced plants (results not shown). The sigmoidal shape of these titration curves indicated that cooperative effects play a role in the binding. It is possible that these include other factors than GT-1.

Binding of nuclear proteins to a $-289/+29$ fragment of the *PR-1a* promoters has recently been reported by Hagiwara *et al.* [13]. From band shift and footprint analyses the authors concluded that a single nuclear protein from non-induced tobacco plants interacted with nucleotides $-184/-172$ and $-65/-57$, whereas an additional footprint was detected at $-31/-24$, overlapping the TATA-box. Moreover, these authors found that the binding to the first two regions was not found in the *N. glutinosa* × *N. debneyi* hybrid that express the *PR-1a* gene constitutively [13]. The protected region detected at position $-184/-172$ exactly overlaps with the box III-like core sequence in fragment G. In tomato the binding of the fruit-specific protein FBF to the *rbcS-3A* promoter correlates with gene inactivation in fruit [21]. Also, for the salt stress-inducible *Ppc1* gene of the common iceplant [7] and the fruit-ripening *E4* gene of tomato [22] it was found that induced expression was accompanied by (partial) dissociation of nuclear proteins from the promoter. Recently, repression of the *L*-asparaginase gene during nodule development in lupin was suggested to be associated with binding of nuclear protein [35]. Furthermore, the GT-1-related SIF-binding site in the promoter of the leaf specific *rps1* gene in tobacco was found to be at least partly responsible for *rps1* gene repression in non-photosynthetic tissues [34].

Since deletion of the far-upstream region of the *PR-1a* promoter until -643 [33] results in loss of inducible expression, it is likely that other interacting factors are necessary for induced expression. Indeed, our band shift experiments revealed that several fragments of the *PR-1a* promoter interact with other nuclear proteins, resulting in complexes with different mobility than those of the slowly migrating GT-1 complexes (e.g. for fragment A see Figs. 2, 3, 5 and 8).

Preliminary experiments showed that two of the three putative GT-1-binding sites present in fragment A, when mutated at a single base pair, result in highly reduced affinity of fragment A for the GT-1-like factor *in vitro*. When these and similar mutations in other putative GT-1-binding sites of the *PR-1a* promoter result in an increase in constitutive gene expression *in vivo*, this may be a hint that negative control plays a role in *PR-1a* gene expression.

Acknowledgements

We would like to thank Dr Johan Memelink, Dr Giancarlo Pasquali and Pieter Ouwerkerk for assistance with the isolation of nuclear extract and their gift of the AS-1 and box II DNAs and the recombinant GT-1 phage. MOGEN Int. is acknowledged for synthesis of oligonucleotide primers. We appreciate Claudia Sombroek for excellent technical assistance. This work was sponsored in part by the Netherlands Organization for Chemical Research (SON) with financial support from the Netherlands Organization for the Advancement of Research (NWO).

References

1. Albrecht H, van de Rhee MD, Bol JF: Analysis of *cis*-regulatory elements involved in induction of a tobacco PR-5 gene by virus infection. Plant Mol Biol 18: 155–158 (1992).
2. Alexander D, Goodman RM, Gutrella M, Glascock C, Weymann K, Friedrich L, Maddox D, Ahl-Goy P, Luntz T, Ward E, Ryals J: Increased tolerance to 2 oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein-1a. Proc Natl Acad Sci USA 90: 7327–7331 (1993).
3. Beilmann A, Pfitzner AJP, Goodman HM, Pfitzner UM: Functional analysis of the pathogenesis-related 1a protein gene minimal promoter region – comparison of reporter gene expression in transient and in stable transfections. Eur J Biochem 196: 415–421 (1991).
4. Bol JF, Linthorst HJM, Cornelissen BJC: Plant pathogenesis-related proteins induced by virus infection. Annu Rev Phytopath 28: 113–138 (1990).
5. Brederode FT, Linthorst HJM, Bol JF: Differential induction of acquired resistance and PR gene expression in a tobacco by virus infection, ethephon treatment, UV light and wounding. Plant Mol Biol 17: 1117–1125 (1991).
6. Cornelissen BJC, Horowitz J, van Kan JAL, Goldberg RB, Bol JF: Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. Nucl Acids Res 15: 6799–6811 (1987).
7. Cushman JC, Bohnert HJ: Salt stress alters A/T-rich DNA-binding factor interactions within the phosphoenolpyruvate carboxylase promoter from *Mesembryanthemum crystallinum*. Plant Mol Biol 20: 411–424 (1992).
8. Elliott RC, Pedersen TJ, Fristensky B, White MJ, Dickey LF, Thompson WF: Characterization of a single copy gene encoding ferredoxin I from pea. Plant Cell 1: 681–690 (1989).
9. Gilmartin PM, Memelink J, Hiratsuka K, Kay SA, Chua N-H: Characterization of a gene encoding a DNA binding protein with specificity for a light-responsive element. Plant Cell 4: 839–849 (1992).
10. Green PJ, Kay SA, Lam E, Chua N-H: *In vitro* DNA footprinting. In: Gelvin SB, Schilperoort RA (eds) Plant Molecular Biology Manual B11: 1–22. Kluwer Academic Publishers, Dordrecht, Netherlands (1989).
11. Green PJ, Kay SA, Chua N-H: Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the *rbc-3a* gene. EMBO J 6: 2543–2549 (1987).
12. Green PJ, Yong MH, Cuozzo M, Kano-Murakami Y, Silverstein P, Chua N-H: Binding site requirements for pea nuclear protein factor GT-1 correlate with sequences required for light-dependent transcriptional activation of the *rbcs-3a* gene. EMBO J 13: 4035–4044 (1988).
13. Hagiwara H, Matsuoka M, Ohshima M, Watanabe M, Hosokawa D, Ohashi Y: Sequence-specific binding of protein factors to 2 independent promoter regions of the acidic tobacco pathogenesis-related-1 protein gene (PR-1). Mol Gen Genet 240: 197–205 (1993).
14. Jefferson RA: Assaying chimeric genes in plants: the *gus* gene fusion system. Plant Mol Biol Rep 5: 387–405 (1987).
15. Kay SA, Keith B, Shinozaki K, Chye M-L, Chua N-H: The rice phytochrome gene: structure, autoregulated expression, and binding of GT-1 to a conserved site in the 5′ upstream region. Plant Cell 1: 351–360 (1989).
16. Kyozuka J, Olive M, Peacock JW, Dennis ES, Shimamoto K: Promoter elements required for developmental expression of the maize *Adh1* gene in transgenic rice. Plant Cell 6: 799–810 (1994).
17. Lam E, Benfey PN, Gilmartin PM, Fang RX, Chua N-H: Site specific mutations alter *in vitro* factor binding and change promoter expression patterns in transgenic plants. Proc Natl Acad Sci USA 86: 7890–7894 (1989).
18. Lawton MA, Dean SM, Dron M, Kooter JM, Kragh KM, Harrison MJ, Yu L, Tanguay L, Dixon RA, Lamb CJ: Silencer region of a chalcone synthase promoter contains multiple binding sites for a factor, SBF-1, closely related to GT-1. Plant Mol Biol 16: 235–249 (1991).

19. Linthorst HJM: Pathogenesis-related proteins of plants. Crit Rev Plant Sci 10: 123–150 (1991).
20. Liu X, Lam E: Two binding sites for the plant transcription factor ASF-1 can respond to auxin treatments in transgenic tobacco. J Biol Chem 269: 668–675 (1994).
21. Meier I, Gruissem W, Fahl E, Gröning B: Organ-specific differential transcriptional activity of *rbcS* promoters in tomato. Abstract, 4th International Congress of Plant Molecular Biology, Amsterdam, p. 250 (1994).
22. Montgomery J, Goldman S, Deikman J, Margossian L, Fischer RL: Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. Proc Natl Acad Sci USA 90: 5939–5943 (1993).
23. Ohshima M, Harada N, Matsuoka M, Ohashi Y: The nucleotide sequence of pathogenesis-related (PR) 1b protein gene of tobacco. Nucl Acids Res 18: 181 (1990).
24. Ohshima M, Harada N, Matsuoka M, Ohashi Y: The nucleotide sequence of pathogenesis-related (PR) 1c protein gene of tobacco. Nucl Acids Res 18: 182 (1990).
25. Ohshima M, Itoh H, Matsuoka M, Murakami T, Ohashi Y: Analysis of stress-induced or salicylic acid-induced expression of the pathogenesis-related 1a protein gene in transgenic tobacco. Plant Cell 2: 95–106 (1990c).
26. Payne G, Parks TD, Burkhart W, Dincher S, Ahl P, Métraux JP, Ryals J: Isolation of the genomic clone for pathogenesis-related protein 1a from *Nicotiana tabacum* cv. Xanthi-nc. Plant Mol Biol 11: 89–94 (1988).
27. Pfitzner UM, Pfitzner AJP, Goodman HM: DNA sequence analysis of a PR-1a gene from tobacco: molecular relationship of heat shock and pathogen responses in plants. Mol Gen Genet 211: 290–295 (1988).
28. Schindler U, Cashmore AR: Photoregulated gene expression may involve ubiquitous DNA binding proteins. EMBO J 9: 3415–3427 (1990).
29. Stockhaus J, Eckes P, Rocha-Sosa M, Schell J, Willmitzer L: Analysis of *cis*-acting sequences involved in the leaf-specific expression of a potato gene in transgenic plants. Proc Natl Acad Sci USA 84: 7943–7947 (1987).
30. Uknes S, Dincher S, Friedrich L, Negrotto D, Williams S, Thompsontaylor H, Potter S, Ward E, Ryals J: Regulation of pathogenesis-related protein-1a gene expression in tobacco. Plant Cell 5: 159–169 (1993).
31. van de Rhee MD, Bol JF: Induction of the tobacco PR-1a gene by virus infection and salicylate treatment involves an interaction between multiple regulatory elements. Plant J 3: 71–82 (1993).
32. van de Rhee MD, Lemmers R, Bol JF: Analysis of regulatory elements involved in stress-induced and organ-specific expression of tobacco acidic and basic β-1,3-glucanase genes. Plant Mol Biol 21: 451–461 (1993).
33. van de Rhee MD, van Kan JAL, Gonzalez-Jaen MT, Bol JF: Analysis of regulatory elements involved in the induction of two tobacco genes by salicylate treatment and virus infection. Plant Cell 2: 357–366 (1990).
34. Villain P, Clabault G, Macke R, Zhou D-X: Sif binding site is related to but different from the light-responsive GT-1 binding site and differentially represses the spinach *rps1* promoter in transgenic tobacco. J Biol Chem 269: 16626–16630 (1994).
35. Vincze E, Reeves JM, Lamping E, Farnden KJF, Reynolds PHS: Repression of the *L*-asparaginase gene during nodule development in *Lupinus angustifolius*. Plant Mol Biol 26: 303–311 (1994).
36. Woloshuk CP, Meulenhoff EJS, Sela-Buurlage M, van den Elzen PJM, Cornelissen BJC: Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell 3: 619–628 (1991).