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Analysis of Regulatory Elements Involved in the Induction of Two Tobacco Genes by Salicylate Treatment and Virus Infection

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Tobacco genes encoding the PR-1a protein and a glycine-rich protein are expressed after treatment of plants with salicylate or infection with tobacco mosaic virus. Upstream sequences of these genes were fused to reporter genes, and these constructs were used to transform tobacco. Upstream sequences of the PR-1a gene of 689 base pairs or longer were sufficient for induction of the reporter gene in tobacco mosaic virus-inoculated leaves, systemically induced leaves from infected plants, and leaves treated with salicylate. No such induction was found with upstream sequences of 643 base pairs or shorter of the PR-1a gene. When the PR-1a upstream sequence from nucleotides −625 to −902 was fused to the cauliflower mosaic virus 35S core promoter, a construct was obtained that conferred tobacco mosaic virus and salicylate inducibility to the reporter gene in transgenic plants. This confirmed the localization of tobacco mosaic virus- and salicylate-responsive elements between positions −643 and −689 in the PR-1a promoter. With the glycine-rich protein gene, an upstream sequence of 645 base pairs was sufficient for tobacco mosaic virus and salicylate inducibility of the reporter gene, whereas constructs containing 400 base pairs or fewer of the glycine-rich protein promoter were largely inactive.

INTRODUCTION

The necrotic infection of plants with pathogens triggers the synthesis of a number of host proteins that are believed to be involved in various defense mechanisms, collectively known as "systemically acquired resistance" (Collinge and Slusarenko, 1987; Bol and Van Kan, 1988). These proteins include enzymes involved in biosynthesis of aromatic compounds, peroxidases, proteinase inhibitors, cell wall components, and the "pathogenesis-related" (PR) proteins. In virus-infected plants, the induction of PR proteins has been studied in most detail, but the induction of other classes of proteins by this type of infection has been reported as well (Van Loon, 1982). The PR proteins induced in Samsun NN tobacco after infection with tobacco mosaic virus (TMV) include acidic PR-1 proteins (PRs 1a, 1b, and 1c) of unknown function, acidic β-1,3-glucanases (PRs 2, N, and O), acidic chitinases (PRs P and Q), and an acidic thaumatin-like protein (PR-S). Basic isoforms of these four classes of acidic PR proteins have been identified in TMV-infected tobacco (Bol and Van Kan, 1988; Bol et al., 1990). In addition to TMV infection, the genes encoding acidic and basic PRs are differentially induced by a number of chemicals, such as ethylene and salicylate. Moreover, the genes for acidic and basic PRs show differences in tissue-specific expression, and their induction is light dependent (for references, see Bol et al., 1990). Thus, an analysis of the promoter regions of these genes may reveal a mosaic of *cis*-acting elements and shed light on the regulation of other classes of stress-induced plant genes as well.

We cloned cDNAs to a number of mRNAs that are strongly induced in Samsun NN tobacco after TMV infection (Hooft van Huijsduijnen et al., 1986). These clones were used as probes to isolate corresponding genes from a genomic library of Samsun NN tobacco. We reported the complete sequence of the PR-1a gene and two PR-1 pseudogenes (Cornelissen et al., 1987), two active PR-S genes (Van Kan et al., 1989), and two genes encoding a glycine-rich protein (GRP) that does not show homology to any of the known PR proteins (Van Kan et al., 1988). In addition, the sequence of the PR-1a gene was reported by several other groups (Ohshima et al., 1987; Payne et al., 1988; Pfitzner et al., 1988). The PR-1a and GRP genes are strongly induced by spraying tobacco with a 5 mM salicylate solution, whereas the PR-S genes are weakly induced by this treatment. A comparison of the putative promoter regions of the PR-1a, GRP, and PR-S genes revealed only limited similarities in the first 100 bp upstream of the respective transcription initiation sites (Van Kan et al., 1989). Here we report an analysis of *cis*-acting elements involved in the induction of the PR-1a and GRP genes by salicylate treatment and TMV infection. The

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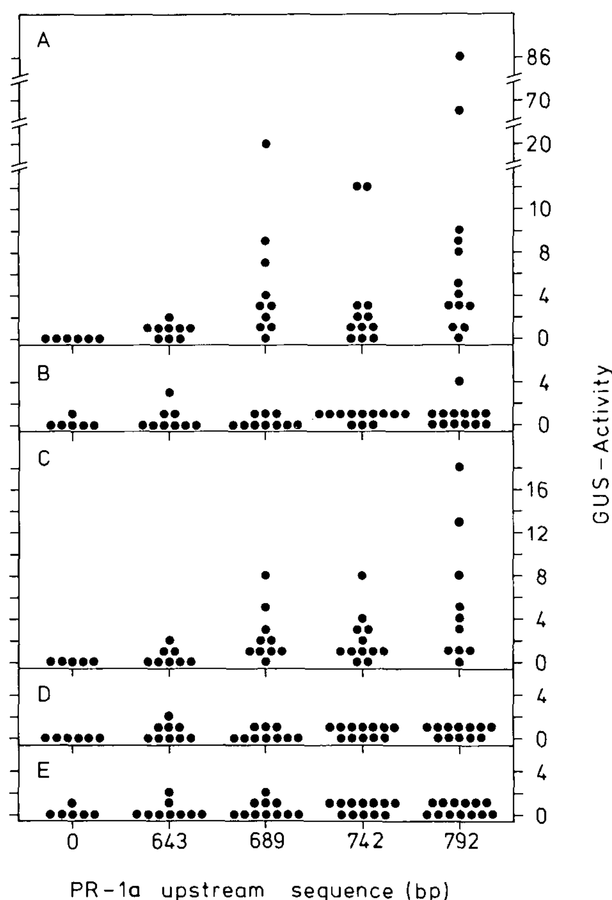

Figure 1. Induction of GUS Activity in Tobacco Transformed with PR-1a/GUS Gene Fusions.

Tobacco plants were transformed with the indicated PR-1a upstream sequences fused to the GUS gene, and leaf discs of these plants were assayed for GUS activity after various treatments. For each construct, 6 to 13 plants were tested. The dots represent the level of GUS activity in the individual primary transformed plants.
(A) Leaf discs taken from TMV-inoculated leaves.
(B) Leaf discs taken from mock-inoculated leaves.
(C) Leaf discs from noninoculated leaves floated on water containing 1 mM salicylate.
(D) Leaf discs from noninoculated leaves floated on water.
(E) Leaf discs from noninoculated leaves assayed immediately after punching.

upstream sequences of the PR-1a gene and an active GRP gene were fused to β-glucuronidase (GUS) and chloramphenicol acetyltransferase (CAT) reporter genes, respectively. Transient expression assays in tobacco protoplasts showed no induction of the GRP/CAT construct by addition of salicylate to the medium. Therefore, the constructs were used to transform tobacco, and transgenic plants were assayed for induction of the reporter genes by salicylate treatment and TMV infection.

RESULTS

Analysis of the PR-1a Promoter

A fragment of the PR-1a gene (Cornelissen et al., 1987) containing the sequence of nucleotides −902 to +29 was fused to the GUS gene in the transformation vector pBI101. Deletions were made with Bal31 starting from position −902 and ending at positions −792, −742, −689, −643, −516, and −436 of the PR-1a upstream sequence. In addition, a restriction fragment containing nucleotides −287 to +29 of the PR-1a gene was fused to the GUS gene. These constructs were used to transform tobacco, and transgenic plants that expressed the selectable marker gene (NPTII) were regenerated. These plants were called PRB plants. Leaf discs were punched from the plants, and GUS activity was measured either directly after punching (zero time) or after flotation of the discs for 24 hr on water or water containing 1 mM salicylate. In addition, GUS activity was measured in leaf discs taken from mock-inoculated or TMV-inoculated leaves. For each promoter construct, 9 to 20 independent transformants were assayed. A considerable variability in expression levels of the introduced gene was found, as has also been observed by others (e.g., Dean et al., 1988). This is illustrated in Figure 1, which shows the GUS activity in individual transformants containing PR-1a upstream sequences ranging from 643 bp to 792 bp. Table 1 shows the average GUS activity observed in plants containing PR-1a upstream sequences with a length of 287 bp to 902 bp. In leaf discs from mock-inoculated leaves, and leaf discs assayed im-

Table 1. Induction of GUS Activity in PRB plants by Salicylate Treatment and TMV Infection[a]

PR-1a Upstream Sequence[b]	*n*	Zero Time	Water	Salicylate	Mock	TMV
bp						
902	20	0.6	1.1	11.3	0.7	51.3
792	13	0.5	0.8	4.8	0.8	15.5
742	12	0.5	0.7	2.1	0.8	3.2
689	10	0.3	0.5	2.3	0.3	4.9
643	10	0.9	1.0	1.0	0.8	1.1
516	9	1.0	0.7	1.2	1.0	0.6
436	9	1.2	1.2	1.0	1.0	1.3
287	9	0.9	1.6	1.2	1.5	0.8
0	6	0.2	0.2	0.2	0.2	0.1

[a] Leaf discs from noninoculated PRB plants were assayed for GUS activity directly after punching (zero time), or after flotation for 24 hr on water (Water) or water containing 1 mM salicylate (Salicylate). In addition, leaf discs were assayed from mock-inoculated (Mock) or TMV-inoculated (TMV) leaves. The average GUS activity in *n* plants is given.
[b] Upstream sequence of 0 bp: control plants transformed with the vector pBI101.

mediately after punching or after floating for 24 hr on water, no significant GUS activity was observed. Moreover, no significant induction of GUS activity by salicylate treatment or TMV infection was observed in leaf discs from 37 plants containing 287 bp to 643 bp of the PR-1a upstream sequences (Table 1). On the other hand, with the plants containing 689 bp to 902 bp of the PR-1a promoter, a significant induction of GUS activity by salicylate treatment or TMV infection was observed in more than half of the plants in each group. On average, the induction by TMV infection was somewhat higher than the induction by salicylate treatment (Table 1). The inducibility of the GUS gene by salicylate and TMV observed in the primary transformants was reproducibly found in the F1 progeny of these plants (results not shown). For a number of PRB643 and PRB689 plants, it was confirmed by DNA gel blot hybridization that, in addition to the selectable marker gene, the GUS gene was present in the genome of these plants (results not shown). This demonstrates that the lack of inducibility of GUS activity in the PRB643 plants is not due to the absence of the reporter gene.

Systemic Induction of the PR-1a Gene

After inoculation of the lower leaves of a Samsun NN tobacco plant with TMV, the PR-1a genes are induced in the primary infected leaves as well as in the virus-free noninoculated upper leaves (Cornelissen et al., 1986). This indicates that the long-distance signal pathway includes a mobile compound that migrates from the lower to the upper leaves. To investigate a possible difference between *cis*-acting elements of the PR-1a promoter responding to local infection and systemic induction, GUS activity was measured in the virus-free upper leaves of PRB plants, the lower leaves of which had been inoculated with TMV. Figure 2 shows the average GUS activity in a number of transformants containing 643 bp to 902 bp of the PR-1a upstream sequence. For each construct, eight F1 plants derived from two independent primary transformants were tested. GUS activity was measured before inoculation, in the primary inoculated leaves, and in the virus-free upper leaves. As a control, it was checked that mock inoculation did not induce GUS activity (Figure 2A). The systemic induction of GUS activity by TMV infection closely paralleled the induction in primary inoculated leaves, although the average level was somewhat lower (Figure 2B). Systemic induction of the reporter gene was obtained with 689 bp or longer upstream sequences of the PR-1a gene, whereas no such induction was obtained with a construct containing 643 bp of the PR-1a promoter. Also, PRB plants with promoter sequences shorter than 643 bp did not show systemic induction of GUS activity after TMV infection (result not shown).

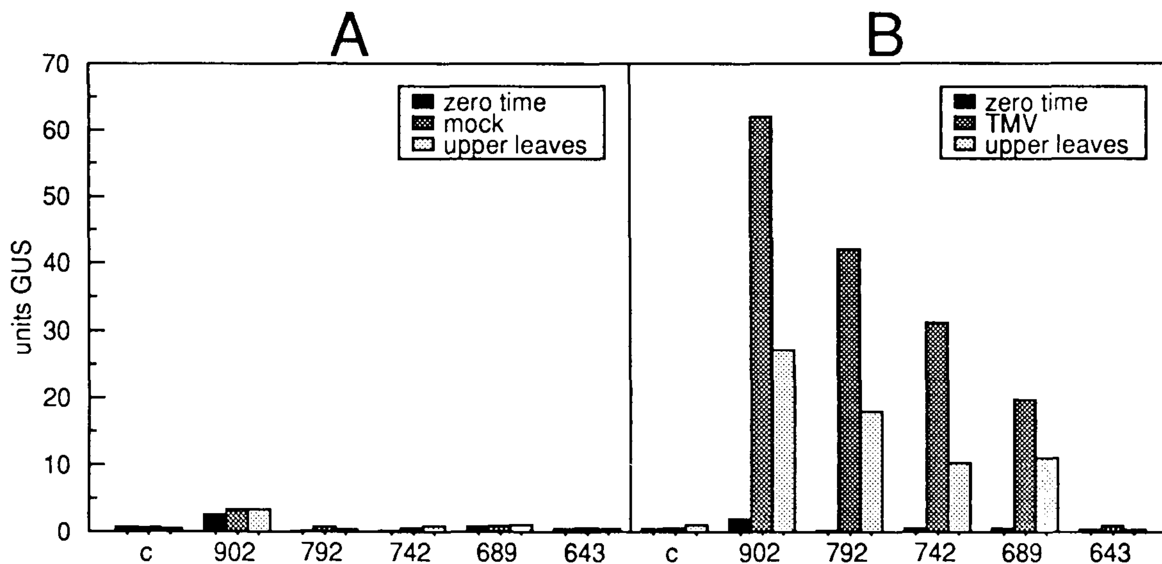

Figure 2. Local and Systemic Induction of GUS Activity in PRB Plants after TMV Infection.

Tobacco plants were transformed with PR-1a upstream sequences of the indicated length (in base pairs) fused to the GUS gene.
(A) Mock-inoculated lower leaves of transformed plants.
(B) TMV-inoculated lower leaves of transformed plants.
GUS activity was assayed in leaf discs taken before inoculation (zero time), taken from inoculated leaves [**(A)**, Mock; **(B)**, TMV], or taken from noninoculated upper leaves. For each PR-1a construct, the average GUS activity in eight F1 plants derived from two independent primary transformants is given. c, nontransformed control plants.

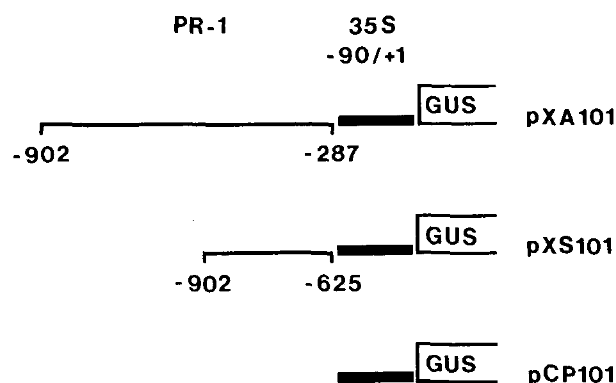

Figure 3. Schematic Representation of PR-1a/35S/GUS Fusions.

In pXA101, the GUS gene in the transformation vector pBI101 is preceded by the CaMV 35S core promoter (nucleotides −90 to +1) and the PR-1a upstream sequence of nucleotides −902 to −287. In pXS101, the PR-1a upstream sequence is reduced to nucleotides −902 to −625, whereas, in pCP101, the PR-1a sequence is absent.

Fusions of PR-1a Sequences with the 35S Core Promoter

The results indicated that salicylate- and TMV-responsive elements are located between positions −643 and −689 in the PR-1a upstream sequence. To confirm this observation, various upstream sequences of the PR-1a gene were fused to the 35S core promoter (nucleotides −90 to +1) of cauliflower mosaic virus. Figure 3 shows schematically that construct pXA101 contains the PR-1a sequence from nucleotide −287 to −902, whereas pXS101 contains the sequence from −625 to −902. As a control, pCP101 contained only the 35S core promoter fused to the GUS gene. These three constructs were used to transform tobacco, and, for each construct, 10 to 14 primary transformants were assayed for induction of GUS activity by salicylate treatment and TMV infection. Table 2 lists the average levels of GUS activity in the various samples of the plants. No significant induction of GUS was observed in any of the pCP101 control plants, whereas, in the pXA101 and pXS101 plants, the reporter gene was clearly induced by salicylate treatment and TMV infection. This induction is further illustrated in Figure 4, which shows the GUS activity in pXA101 and pXS101 plants with the highest, an intermediate, and the lowest expression levels.

Analysis of the GRP Promoter

Fragments containing 645 bp, 400 bp, 135 bp, and 114 bp of the upstream sequence of the GRP gene in clone gGRP-8 (Van Kan et al., 1988) were fused to the CAT reporter gene. At the 3′ end, all fragments terminated at position +8 in the GRP leader sequence. The constructs were used to transform tobacco, and the resulting transgenic plants were named PRC645 (10 plants), PRC400 (5 plants), PRC135 (10 plants), and PRC114 (5 plants). Induction of the CAT gene in these plants was assayed by floating leaf discs for 24 hr on water or water containing 1 mM salicylate. Table 3 lists the average levels of CAT activity measured in these leaf discs, and Figure 5 shows the results of CAT assays done with representative plants from the four groups of transformants. In general, the PRC114, PRC135, and PRC400 plants showed little or no induction of CAT activity by salicylate (Figure 5, lanes 6 to 11). However, one PRC135 plant (PRC135-7) showed an exceptionally high induction of the reporter gene by salicylate (Figure 5, lanes 1 and 2). In contrast to all other transformants, this plant showed an abnormal phenotype. The distance between internodes was increased and the leaves were yellow-green.

In one of the PRC645 plants (PRC645-4), the CAT gene was not inducible by salicylate. All other PRC645 plants showed CAT activities comparable with those shown in lanes 3 to 5 of Figure 5. The background in leaf discs assayed immediately after punching was rather low (lane 3). Flotation on water induced a significant CAT activity (lane 4). This may be due to the wounding involved in making the leaf discs. However, when the leaf discs were floated on the salicylate solution, the CAT activity was 3 times to 7 times higher than in the water-treated control (Figure 5, lane 5; Table 3).

Seeds from two PRC400 plants (PRC400-4 and -5) and three PRC645 plants (PRC645-1, -3 and -6) were used to grow kanamycin-resistant progeny plants. These plants were assayed for induction of the CAT gene by TMV infection, using salicylate treatment as a control. Table 4 lists the average level of CAT activity measured in samples of these plants. In the PRC400 plants, salicylate and TMV induced no detectable CAT activity. In the PRC645 progeny plants, the background of CAT activity (zero time sample) was higher than in the primary transformants. This may be due to a difference in growing conditions of the plants. Compared with the water-treated control, salicylate

Table 2. Induction of GUS Activity in Plants Transformed with pXA101, pXS101, and pCP101[a]

Transformant	*n*	Zero Time	Water	Salicylate	Mock	TMV
pXA101	10	0.6	0.8	2.3	0.8	10.3
pXS101	10	0.5	0.8	2.3	1.0	3.7
pCP101	14	0.1	0.1	0.3	0.1	0.1

[a] The structure of plasmids pXA101, pXS101, and pCP101 is shown in Figure 3. Experimental conditions are given in a footnote to Table 1.

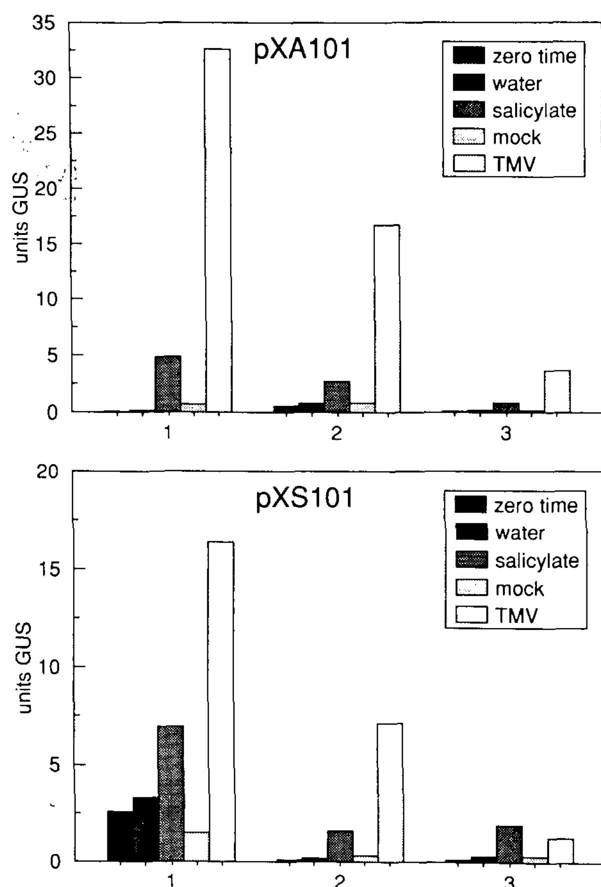

Figure 4. Induction of GUS Activity in Tobacco Transformed with PR-1a/35S/GUS Fusions.

Plants were transformed with constructs pXA101 and pXS101 shown schematically in Figure 3. Leaf discs from noninoculated plants were assayed for GUS activity directly after punching (zero time) or after flotation for 24 hr on water (water) or water containing 1 mM salicylate (salicylate). In addition, leaf discs were assayed from mock-inoculated (mock) and TMV-inoculated (TMV) leaves. For each construct, the GUS activity is shown for the plant with the highest expression level (1), an intermediate expression level (2), and the lowest expression level (3).

treatment caused a threefold to fourfold increase of CAT activity, as was observed before. Mock inoculation of PRC645 plants did not induce the reporter gene, but TMV infection resulted in a sevenfold to eightfold increase in the level of CAT activity. Data obtained with a representative PRC645 plant are shown in Figure 6. Together, the results demonstrate that elements between −400 and −645 of the GRP upstream sequence are important to induction of the reporter gene by both salicylate treatment and TMV infection.

DISCUSSION

Genes encoding PR-1-like proteins are highly conserved in both dicotyledonous and monocotyledonous plants (Nassuth and Sänger, 1986; White et al., 1987), and PR-1 may constitute more than 1% of the soluble leaf protein when plants are subjected to various stress conditions (Antoniw and Pierpoint, 1978). In addition to a hypersensitive response to pathogens, PR-1 proteins are known to be induced by a wide variety of abiotic agents, including plant hormones such as ethylene, abscisic acid, and cytokinin; aromatic compounds like benzoic acid, salicylic acid, and acetylsalicylic acid; amino acid derivatives; polyacrylic acid; barium and manganese salts; and elicitors obtained from fungal and bacterial cell cultures (for references see Van Loon, 1982, 1985; Bol, 1988). Inducers triggering the expression of tobacco GRP genes have been studied in less detail. It is known that GRP expression is leaf specific and light dependent and that the GRP genes are strongly induced by ethylene (Memelink, 1988). The function of PR-1 and GRP is not known. It has been suggested that these salicylate-inducible proteins are involved in the salicylate-induced resistance of tobacco to virus infection. However, constitutive expression of PR-1 or GRP in transgenic tobacco did not reduce the susceptibility of the plants to infection with TMV or alfalfa mosaic virus (Linthorst et al., 1989). Glycine-rich proteins, induced by wounding, water stress, or plant hormones, have been characterized at the molecular level in petunia (Condit and Meagher, 1987), bean (Keller et al., 1988), maize (Gomez et al., 1988), and rice (Mundy and Chua, 1988). The bean GRP has been shown to be a cell wall component, associated with the vascular system (Keller et al., 1988). By analogy, the TMV-induced tobacco GRP may be a cell wall component.

Table 3. Induction of CAT Activity in PRC Plants by Salicylate Treatment[a]

GRP Upstream Sequence[b]	*n*	Water	Salicylate
bp			
645	10	37.7	115.9
400	5	0.1	0.3
135	10	0.0	0.4
114	5	0.0	0.1
0	3	0.0	0.0

[a] Leaf discs from PRC plants were assayed for CAT activity after flotation for 24 hr on water (Water) or water containing 1 mM salicylate (Salicylate). The average of CAT activity in *n* plants is given.
[b] Upstream sequence of 0 bp: control plants transformed with pAGS129 containing the CAT gene.

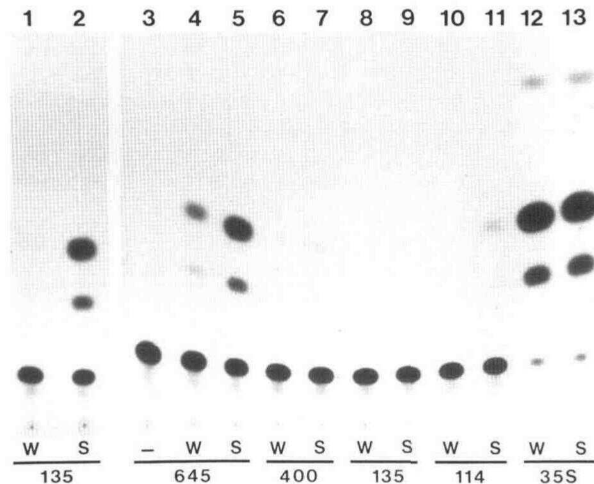

Figure 5. Induction of CAT Activity in Tobacco Transformed with GRP/CAT Gene Fusions.

Figures below the bars give the number of base pairs of the GRP upstream sequences that were fused to the CAT gene. Leaf discs of plants PRC135-7 (lanes 1 and 2), PRC645-1 (lanes 3 to 5), PRC400-4 (lanes 6 and 7), PRC135-8 (lanes 8 and 9), and PRC114-2 (lanes 10 and 11) were assayed for CAT activity either directly (lane 3), or after flotation on water (lanes W) or water containing 1 mM salicylate (lanes S). As a control, the CAT gene was fused to the CaMV 35S promoter (lanes 12 and 13).

Studies on signals and transduction mechanisms for activation of plant defense genes represent an active area of research (for a review, see Lamb et al., 1989). For the potato proteinase inhibitor II gene, evidence has been obtained that an upstream sequence of 1 kb contains sequences necessary for wound-inducible expression (Thornburg et al., 1987; An et al., 1989). A functional analysis of the promoter of the bean chalcone synthase gene suggested the location of an activator between the TATA-box and −173 and an upstream silencer between −173 and −326, modulating the induction of the gene by elicitor and glutathione (Dron et al., 1988). Stimulation of transcription by glutathione was accompanied by the induction of three DNase I hypersensitive sites between −53 and +79, suggesting the location of binding sites for transacting factors in this region (Lawton et al., 1990). Our results indicate that more extensive upstream sequences are required for the induction of tobacco PR-1 and GRP genes by microbial attack and abiotic agents. Figure 7 shows a schematic representation of various elements that have been recognized in the upstream sequences of the PR-1a and GRP genes. The finding that the sequence of −689 to +29 of the PR-1a gene is sufficient for induction of the reporter gene by salicylate treatment and TMV infection indicates that the 36-bp direct repeat upstream of position −689 may not be involved in these processes. Because constructs with 643-bp or smaller fragments of the upstream sequence were inactive, the region between −689 and −643 probably contains elements important to the induction of the PR-1a gene by the two treatments. The finding that the sequence from −625 to −902 confers salicylate and TMV inducibility to a fusion of the 35S core promoter and the GUS gene is in agreement with this conclusion. Apparently, regulatory elements involved in the induction of the PR-1a gene by salicylate treatment, by local TMV infection, and by systemic induction in TMV-infected plants either coincide or are clustered in a small region of the PR-1a promoter. The data from Table 1 and Figure 1B suggest a gradual decrease in the inducibility of the reporter gene when the upstream sequence of the PR-1a gene is reduced from 902 bp to 689 bp. This could indicate that elements in this region enhance the induction. However, in view of the wide variation in promoter activity among independent transgenic plants observed in this study and by others (Odell et al., 1987; Sanders et al., 1987; Dean et al., 1988), it is difficult to draw conclusions from quantitative differences observed with PRB plants containing 689 bp to 902 bp of the PR-1a upstream sequences.

Figure 7 illustrates that the GRP upstream sequence is rather rich in direct and inverted repeats, the most prominent being a 64-bp inverted repeat that is almost identical to a similar structure in the upstream sequence of the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene of tobacco (Van Kan et al., 1988). All of these repeats are part of the sequence of −645 to +8 that was found to be required for a reproducible induction of the reporter gene by salicylate treatment and TMV infection. Because transformants containing 400 bp or fewer of the GRP upstream sequence were largely inactive, elements present between −400 and −645 are probably required for efficient induction of the GRP gene by salicylate treatment and TMV infection. Around −540, the sequence TGTGGAAA is found, which is identical to the SV40 core enhancer sequence (ENH in Figure 7). A comparison of the PR-1a and GRP upstream sequences revealed only very limited sequence similarities (Van Kan et al., 1988). Between −689 and −643 of the PR-1a promoter, the sequences TGGAAATTA and GGAAATTA are found, which resemble the SV40 core enhancer to some extent.

Table 4. Induction of CAT Activity in PRC Plants by Salicylate Treatment and TMV Infection[a]

GRP Upstream Sequence	n	Zero Time	Water	Salicylate	Mock	TMV
bp						
645	4	14.3	35.1	122.2	10.7	77.1
400	4	0.1	0.2	0.1	0.0	0.1

[a] Experimental conditions are given in a footnote to Table 1.

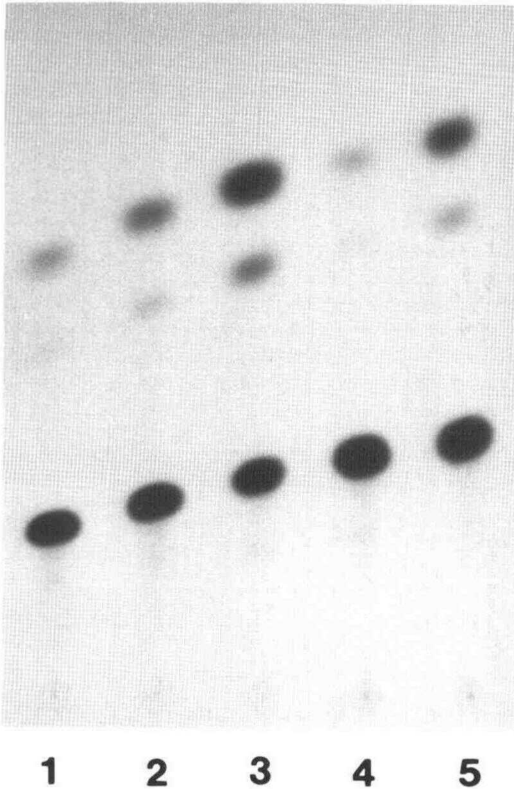

Figure 6. Induction of CAT Activity in PRC645 Plants.

Leaf discs from a healthy PRC645 plant were assayed for CAT activity immediately after punching (lane 1) or after flotation for 24 hr on water (lane 2) or water containing 1 mM salicylate (lane 3). Discs from mock-inoculated (lane 4) or TMV-inoculated PRC645 leaves (lane 5) were assayed immediately after punching.

A comparison of this region of the PR-1a promoter with upstream sequences of other TMV-inducible tobacco genes did not reveal any significant sequence similarity (Van Kan et al., 1989).

Recently, regulatory elements have been analyzed of an ethylene-inducible bean PR gene encoding a chitinase (Broglie et al., 1989). The results pointed to the location of an activator between −575 and −422 and the location of ethylene-responsive elements between −422 and −195 in the upstream sequence of this chitinase gene. It could be that, also in the tobacco GRP gene, the sequence from −400 to −645 contains an activator, whereas salicylate- and TMV-responsive elements are located closer to the TATA box. In this case, the unusual inducibility of the CAT gene in the PRC135-7 plant (Figure 5, lane 2) could be explained by integration of the chimeric gene near an enhancer sequence in the recipient DNA. Alternatively, the stress condition caused by the abnormal phenotype of this plant could have played a role.

In addition to PR-1 and GRP genes, we have cloned seven other classes of tobacco genes that are strongly induced by TMV infection (Bol et al., 1990). It will be interesting to compare the TMV-responsive elements of these nine classes of genes and to analyze transacting factors involved in their coordinate induction by TMV infection.

METHODS

PR-1a Promoter Constructs

The numbering of the upstream sequence of the PR-1a gene that we reported previously (Cornelissen et al., 1987) was corrected according to data published more recently (Payne et al., 1988; Pfitzner et al., 1988). A HindIII site at position +856 of the PR-1a genomic clone (Cornelissen et al., 1987) was used as the starting point to remove the coding region by Bal31 digestion up to nucleotide +30 in the leader sequence. The digestion was followed by addition of a BamHI linker.

An XhoI site at position −902 in the upstream sequence and the generated BamHI site were used to produce a fragment from the truncated clone containing nucleotides −902 to +29 of the PR-1a gene. This fragment was inserted upstream of the GUS gene in the SalI/BamHI-digested transformation vector pBI101 (Jefferson et al., 1987), creating construct PRB902. To obtain smaller PR-1a promoter fragments, a series of deletions were made with Bal31, starting from position −902 in PRB902, ending at positions −792, −742, −689, −643, −516, and −436. HindIII linkers were added to the truncated ends, and, after cutting with HindIII and BamHI, the PR-1a promoter fragments were fused to the GUS gene by ligation into pBI101 digested with HindIII and

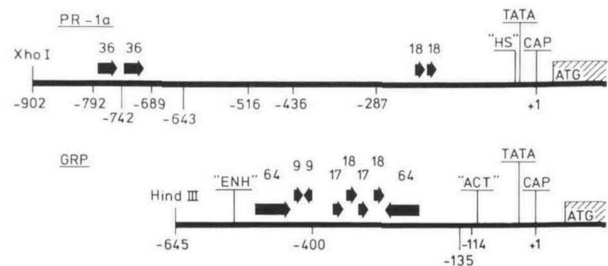

Figure 7. Structural Features of the PR-1a and GRP Upstream Sequences.

The 5′ region of the coding sequences of the PR-1a and GRP sequences are indicated by hatched boxes. Arrows represent direct or inverted repeats of the indicated length. Figures below the bars give the 5′ end of deletion mutants discussed in the text. Other features are: CAP, transcription initiation site (+1); TATA, TATA box; HS, box resembling the heat-shock consensus sequence; ACT, box that has been identified as an activator sequence in several plant genes; ENH, box that is identical to the SV40 core enhancer sequence. Data are from Cornelissen et al. (1987) and Van Kan et al. (1988).

BamHI. The resulting constructs were named PRB792, PRB742, PRB689, PRB643, PRB516, and PRB436, the figure giving the number of base pairs of the PR-1a upstream sequence present in the construct. Another promoter fragment was obtained by using an AccI site at position −287 of the PR-1a promoter. After digestion with AccI and addition of a HindIII linker, a HindIII/BamHI fragment was obtained containing nucleotides −287 to +29 of the PR-1a gene. This fragment was ligated into pBI101, yielding construct PRB287. Leaf discs of *Nicotiana tabacum* cv Samsun NN were transformed with the PR-1a constructs and with pBI101 (PRB0) as a negative control, and transgenic plants were regenerated as described below.

Chimeric PR-1a/35S Promoter Constructs

A fragment containing the cauliflower mosaic virus (CaMV) 35S core promoter (nucleotides −90 to +1) was obtained by digesting pMOG181 (supplied by MOGEN International, Leiden) with EcoRV and BamHI. The transformation vector pBI101 was digested with XbaI, treated with Klenow polymerase to generate blunt ends, and digested with BamHI. Subsequently, the 35S promoter fragment was ligated into this vector, creating pCP101. The PR-1a genomic clone was digested with XhoI and SspI to obtain a promoter fragment containing nucleotides −902 to −625, whereas digestion with XhoI and AccI was used to isolate a fragment containing nucleotides −902 to −287. After addition of SalI linkers, the PR-1a promoter fragments were cloned upstream of the 35S promoter element in pCP101 digested with SalI. The orientation of the PR-1a fragments was checked by restriction enzyme analysis, and the constructs were named pXA101 (nucleotides −902 to −287) and pXS101 (nucleotides −902 to −625). Tobacco leaf discs were transformed with pCP101, pXA101, and pXS101 as described below.

GRP Promoter Constructs

Studies on the GRP promoter were done with a HindIII restriction fragment containing nucleotides −645 to +155 of the genomic clone gGRP-8 (Van Kan et al., 1988). The sequence from +9 to +155, including the ATG initiation codon of the GRP reading frame, was removed by Bal31 digestion. The resulting fragment was used to generate a nested set of subclones with the 5′-terminal sequence reduced to −400, −135, and −114 by making use of EcoRV, HaeIII, and AvaII restriction sites occurring at these positions, respectively. Using ClaI linkers, these fragments were fused to the CAT gene containing a polyadenylation signal from the nopaline synthetase gene (Tnos). In control experiments, the GRP sequence was replaced by a fragment containing the CaMV 35S promoter. The fragments containing the CAT gene, Tnos, and the 35S promoter have been described (Fromm et al., 1985; Van Dun et al., 1987). The GRP/CAT constructs, cloned in pUC8, were inserted in the HindIII site of the transformation vector pAGS129 (Van Dun et al., 1987).

Plant Transformation

Transformation vectors were transferred to *Agrobacterium tumefaciens* by a triparental mating procedure (Van Dun et al., 1987). Leaf discs of *N. tabacum* cv Samsun NN were transformed with the PR-1a and GRP constructs, and transgenic plants were regenerated as described (Horsch et al., 1985). Resistance to kanamycin, conferred by the NPTII gene in the transformation vectors, was used as a selectable marker. Progeny plants from primary transformants were obtained by germinating seeds on a medium containing kanamycin.

Treatment of Transgenic Plants

Discs of 2.5 cm were punched out from leaves of transgenic plants and floated in a Petri dish on water or on a neutralized solution containing 1 mM of salicylate for 24 hr at 26°C. After incubation, the leaf discs were frozen in liquid nitrogen and stored at −20°C until homogenization. For TMV inoculation, carborundum-dusted leaves were rubbed with a suspension of 5 μg/mL TMV (strain WU1) in PEN buffer (10 mM NaH_2PO_4, 1 mM EDTA, 1 mM NaN_3, pH 7.0). Leaf discs were punched out from primary inoculated and virus-free upper leaves at 4 days and 14 days after inoculation, respectively.

CAT and GUS Assays

For CAT assays, each leaf disc was homogenized in 1.5 mL of buffer containing 250 mM Tris/HCl, 5 mM EDTA, pH 7.5. The assay was done with 0.1-mL samples as described (Fromm et al., 1985). CAT activity is expressed in units; 1 unit is the activity converting 1 nmol of chloramphenicol per hour per milligram of extracted protein. For GUS assays, each leaf disc was homogenized in 1.5 mL of a buffer containing 50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarkosyl, 10 mM β-mercaptoethanol. The homogenate was centrifuged for 10 min in an Eppendorf centrifuge, and samples of 5 μL to 20 μL of the supernatant were incubated in a final volume of 0.1 mL containing 50 mM NaH_2PO_4, pH 7.0, 0.1% Triton X-100, 10 mM β-mercaptoethanol, 1 mM *p*-nitrophenylglucuronide, 0.02% NaN_3, and 100 μg/mL BSA. After an incubation of 16 hr at 37°C, the reaction was terminated by the addition of 40 μL of 2.5 M 2-amino-2-methyl-propanediol. Absorbance was measured at 405 nm against a stopped blank reaction to which an identical amount of extract had been added. GUS activity is given in units; 1 unit is the activity converting 1 nmol of *p*-nitrophenylglucuronide per hour per milligram of extracted protein.

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REFERENCES

An, G., Mitra, A., Choi, H.K., Costa, M.A., An, K., Thornburg,

R.W., and Ryan, C.A. (1989). Functional analysis of the 3′ control region of the potato wound-inducible proteinase inhibitor II gene. Plant Cell **1**, 115–122.

Antoniw, J.F., and Pierpoint, W.S. (1978). The purification and properties of one of the "b" proteins from virus-infected tobacco plants. J. Gen. Virol. **39**, 343–350.

Bol, J.F. (1988). Structure and expression of plant genes encoding pathogenesis-related proteins. In Temporal and Spatial Regulation of Plant Genes, D.P.S. Verma and R.B. Goldberg, eds (Vienna: Springer-Verlag), pp. 201–221.

Bol, J.F., and Van Kan, J.A.L. (1988). The synthesis and possible functions of virus-induced proteins in plants. Microbiol. Sci. **5**, 47–52.

Bol, J.F., Linthorst, H.J.M., and Cornelissen, B.J.C. (1990). Plant pathogenesis-related proteins. Annu. Rev. Phytopathol. **28**, in press.

Broglie, K.E., Biddle, P., Cressman, R., and Broglie, R. (1989). Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. Plant Cell **1**, 599–607.

Collinge, D.B., and Slusarenko, A.J. (1987). Plant gene expression in response to pathogens. Plant Mol. Biol. **9**, 389–410.

Condit, C.M., and Meagher, R.B. (1987). Expression of a gene encoding a glycine-rich protein in petunia. Mol. Cell. Biol. **12**, 4273–4279.

Cornelissen, B.J.C., Hooft van Huijsduijnen, R.A.M., Van Loon, L.C., and Bol, J.F. (1986). Molecular characterization of messenger RNAs for "pathogenesis-related" proteins 1a, 1b and 1c, induced by TMV infection of tobacco. EMBO J. **5**, 37–40.

Cornelissen, B.J.C., Horowitz, J., Van Kan, J.A.L., Goldberg, R.B., and Bol, J.F. (1987). Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. Nucl. Acids Res. **15**, 6799–6811.

Dean, C., Jones, J., Favreau, M., Dunsmuir, P., and Bedbrook, J. (1988). Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. Nucl. Acids Res. **16**, 9267–9283.

Dron, M., Clouse, S.D., Dixon, R.A., Lawton, M.A., and Lamb, C.J. (1988). Glutathione and fungal elicitor regulation of a plant defense gene promoter in electroporated protoplasts. Proc. Natl. Acad. Sci. USA **85**, 6738–6742.

Fromm, M., Taylor, R.P., and Walbot, V. (1985). Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc. Natl. Acad. Sci. USA **82**, 5824–5828.

Gomez, J., Sanchez-Martinez, D., Stiefel, V., Rigau, J., Puigdomènech, P., and Pagès, M. (1988). A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. Nature **334**, 262–264.

Hooft van Huijsduijnen, R.A.M., Van Loon, L.C., and Bol, J.F. (1986). cDNA cloning of six mRNAs induced by TMV infection of tobacco and a characterization of their translation products. EMBO J. **5**, 2057–2061.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science **227**, 1227–1231.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6**, 3901–3907.

Keller, B., Sauer, N., and Lamb, C.J. (1988). Glycine-rich cell-wall proteins in bean: Gene structure and association of the protein with the vascular system. EMBO J. **7**, 3625–3633.

Lamb, C.J., Lawton, M.A., Dron, M., and Dixon, R.A. (1989). Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell **56**, 215–224.

Lawton, M.A., Clouse, S.D., and Lamb, C.J. (1990). Glutathione-elicited changes in chromatin configuration in the promoter of the plant defense gene chalcone synthase. EMBO J., in press.

Linthorst, H.J.M., Meuwissen, R.L.J., Kauffmann, S., and Bol, J.F. (1989). Constitutive expression of pathogenesis-related proteins PR-1, GRP, and PR-S in tobacco has no effect on virus infection. Plant Cell **1**, 285–291.

Memelink, J. (1988). Altered gene expression in T-DNA transformed tobacco tissues. Thesis, Leiden University, Leiden, The Netherlands.

Mundy, J., and Chua, N.-H. (1988). Abscisic acid and water-stress induce the expression of a novel rice gene. EMBO J. **7**, 2279–2286.

Nassuth, A., and Sänger, H.L. (1986). Immunological relationship between "pathogenesis-related" leaf proteins from tomato, tobacco and cowpea. Virus Res. **4**, 229–242.

Odell, J.T., Nagy, F., and Chua, N.-H. (1987). Variability in 35S promoter expression between independent transformants. In Plant Gene Systems and Their Biology, L. McIntosh and J. Key, eds (New York: Alan R. Liss, Inc.), Vol. 62, pp. 329–331.

Ohshima, M., Matsuoka, M., Yamamoto, N., Tanaka, Y., Kano-Murakami, Y., Ozeki, Y., Kato, A., Marada, N., and Ohashi, Y. (1987). Nucleotide sequence of the PR-1 gene of *Nicotiana tabacum*. FEBS Lett. **225**, 243–246.

Payne, G., Parks, D., Burkhart, W., Dincher, S., Ahl, P., Metraux, J.P., and Ryals, J. (1988). Isolation of a genomic clone for pathogenesis-related protein 1a from *Nicotiana tabacum* cv. Xanthi-nc. Plant Mol. Biol. **11**, 89–94.

Pfitzner, U.M., Pfitzner, A.J.P., and Goodman, H.M. (1988). DNA sequence analysis of a PR-1a gene from tobacco: Molecular relationships of heat shock and pathogen responses in plants. Mol. Gen. Genet. **211**, 290–295.

Sanders, P.R., Winter, J.A., Zarnason, A.R., Rogers, S.G., and Fraley, R.T. (1987). Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucl. Acids Res. **15**, 1543–1558.

Thornburg, R.W., An, G., Cleveland, T.E., Johnson, R., and Ryan, C.A. (1987). Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyltransferase gene fusion in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA **84**, 744–748.

Van Dun, C.M.P., Bol, J.F., and Van Vloten-Doting, L. (1987). Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic tobacco. Virology **159**, 299–305.

Van Kan, J.A.L., Cornelissen, B.J.C., and Bol, J.F. (1988). A virus-inducible tobacco gene encoding a glycine-rich protein shares putative regulatory elements with the ribulose bisphosphate carboxylase small subunit gene. Mol. Plant-Microbe Int. **1**, 107–112.

Van Kan, J.A.L., Van de Rhee, M.D., Zuidema, D., Cornelissen, B.J.C., and Bol, J.F. (1989). Structure of tobacco genes encoding thaumatin-like proteins. Plant Mol. Biol. **12**, 153–155.

Van Loon, L.C. (1982). Regulation of changes in proteins and enzymes associated with active defense against virus infection.

In Active Defense Mechanisms in Plants, E.K.S. Wood, ed (New York: Plenum Press), pp. 247–273.

Van Loon, L.C. (1985). Pathogenesis-related proteins. Plant Mol. Biol. **4,** 111–116.

White, R.F., Rybicki, E.P., Von Wechmar, M.B., Dekker, J.L., and Antoniw, J.F. (1987). Detection of PR-1 type proteins in *Amaranthaceae*, *Chenopodiaceae*, *Gramineae* and *Solanaceae* by immunoelectroblotting. J. Gen. Virol. **68,** 2043–2048.