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Constitutive Expression of Pathogenesis-Related Proteins PR-1, GRP, and PR-S in Tobacco Has No Effect on Virus Infection

Huub J. M. Linthorst,[a,1] Ralph L. J. Meuwissen,[a] Serge Kauffmann,[b] and John F. Bol[a]

[a]Department of Biochemistry, State University of Leiden, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands
[b]Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, 12, rue du Général Zimmer, 67000 Strasbourg, France

Samsun NN tobacco cells were transformed with chimeric genes for pathogenesis-related (PR) proteins derived from genomic (PR-1a, GRP) or cDNA (PR-S) clones under the transcriptional control of the cauliflower mosaic virus 35S promoter. Regenerated plants were assayed by RNA and protein gel blotting, and plants showing high specific expression of the inserted genes were selected for self-pollination and seed formation. Inspection of second generation transformants showed that constitutive expression of PR-1a, GRP, and PR-S in tobacco in general does not have an effect on the phenotypic appearance of the plants or the expression of other endogenous PR genes. Furthermore, constitutive expression of the above genes does not affect the susceptibility of the plants to infection with tobacco mosaic virus or alfalfa mosaic virus.

INTRODUCTION

Samsun NN tobacco responds to infection with tobacco mosaic virus (TMV) in a hypersensitive way. Small necrotic lesions are formed within 2 to 3 days after inoculation on the inoculated leaves, and the infection usually does not spread to other parts of the plant. However, these noninfected parts appear to have acquired a resistance to the virus when subsequently challenged with TMV. In addition, infection with other pathogens to which the plant reacts in a hypersensitive way with the formation of local necrosis induces resistance to further infection with either virus, fungus, or bacterium (Gianninazzi, 1983). This phenomenon of acquired resistance is paralleled with the appearance of a set of proteins collectively known as pathogenesis-related (PR) proteins (Van Loon, 1982). These proteins appear to have acidic pI values, to be highly protease-resistant, and to be excreted to the intercellular fluid (Van Loon, 1988).

Recently, characterization of cDNA clones and serologic studies have revealed that several of these acidic PR proteins have homologous basic counterparts (Cornelissen et al., 1987; Kauffman et al., 1987; Legrand et al., 1987; Shinshi et al., 1987). These basic proteins are also induced upon TMV infection and are possibly transported to the vacuole (Singh et al., 1987).

In vitro studies have indicated the possible functions of several of these induced proteins. PR proteins P and Q have chitinase activity (Legrand et al., 1987), whereas PR-2, N and O are β-1,3-glucanases (Kauffman et al., 1987), suggesting that these hydrolyzing enzymes could be involved in the degradation of bacterial and fungal cell walls or insect exoskeletons. Indeed, in vitro experiments have shown the inhibitory effect on fungal growth of mixtures of purified chitinase and glucanase from pea (Mauch et al., 1988). PR-S has extensive sequence homology with a maize protein inhibiting the activity of proteinase and insect α-amylase (Richardson et al., 1987), which suggests a possible function in the resistance to insect attack.

The function of the three almost identical PR-1 proteins (PR-1a, 1b, and 1c) is not yet known. Neither is this the case with another protein of which the messenger was found to be induced upon infection with TMV (Hooft van Huijsduijnen et al., 1986b). The sequence of this protein reveals a high percentage of glycine, which, by analogy to other glycine-rich proteins (Condit and Meagher, 1986; Varner and Cassab, 1986), suggests its presence in the cell wall (Van Kan et al., 1988a). PR-1 and the glycine-rich protein (GRP) are the only TMV-induced proteins found so far that are also strongly induced by spraying tobacco with salicylic acid solutions (Hooft van Huijsduijnen et al., 1986b). The observation that treatment of tobacco with salicylic acid renders resistance to TMV and alfalfa mosaic virus (AlMV) infection (White, 1979; Hooft van Huijsduijnen et al., 1986a) prompted us to investigate the possible role of PR-1 and GRP in the acquired resistance of tobacco to virus infection.

[1]To whom correspondence should be addressed. Current address: Gorlaeus Laboratories, State University of Leiden, P.O. Box 9502, 2300 RA Leiden, The Netherlands.

For this study we transformed tobacco with chimeric genes composed of genomic or cDNA coding sequences of PR-1a, GRP, and PR-S under the control of the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter.

RESULTS

Construction of Chimeric PR-1a, GRP, and PR-S Genes

A genomic clone (gPR-1a, Cornelissen et al., 1987) was used for the construction of the chimeric PR-1a gene. After deletion of the upstream promoter region and 5′-noncoding sequence and addition of a HindIII linker immediately in front of the ATG start codon, the coding sequence was inserted between the CaMV 35S promoter and nopaline synthase terminator of the transformation vector. The resulting gene is shown in Figure 1. The PR-1a messenger transcribed from this gene will contain 28 5′-noncoding nucleotides in front of the start codon and possibly terminate somewhere on one of several polyadenylation signals in the 322-nucleotide-long 3′-noncoding region. Payne et al. (1988) found that PR-1a mRNA from Xanthi-nc tobacco is polyadenylated at at least two sites in this nontranslated region. If transcription termination of the transgenic messenger takes place at these internal polyadenylation sites, the resulting messenger will be only one nucleotide shorter than the endogenous PR-1a messengers.

The in vivo expressed GRP gene present on the genomic clone gGRP-8 (Van Kan et al., 1988a) was used for the construction of the chimeric GRP gene. By restriction enzyme digestion and subcloning, a fragment was obtained that contains the coding sequence of GRP present on two exons, a 3′ 341-bp-long noncoding region with several putative polyadenylation signals and a 5′-noncoding sequence, which starts 16 bp in front of the original gGRP-8 cap site. This fragment was cloned between the CaMV promoter and nopaline synthase terminator of the transformation vector (Figure 1). The messenger transcribed from this gene will, therefore, contain a 5′-noncoding sequence of 87 nucleotides that is 35 nucleotides longer than the endogenous GRP messenger.

Finally, a cDNA clone for PR-S was used to construct the chimeric PR-S gene (PROB12, Cornelissen et al., 1986). The messenger from this gene will contain 22 5′-noncoding nucleotides and a 3′-noncoding region derived from the cDNA clone of approximately 160 nucleotides (Figure 1). Although this region contains one AATAAA box about 75 nucleotides upstream of the 3′ end, the clone did not contain a poly(A) stretch. However, transcription termination may also take place at the nopaline synthase terminator in the transformation vector. Also, in this case, the size of the transgenic messenger will probably be only slightly different from that of the endogenous messenger.

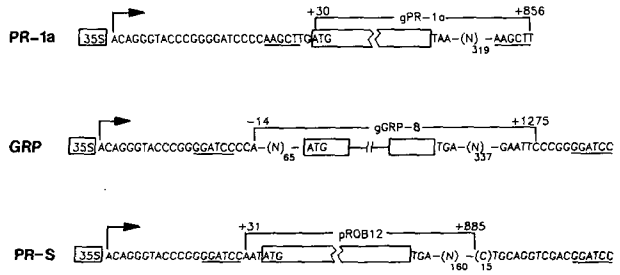

Figure 1. Chimeric Genes for PR-1a, GRP, and PR-S.

Schematic representation of the coding sequences for the chimeric genes constructed behind the CaMV 35S promoter (boxed) with the starts of the transcripts indicated by arrows. The open reading frames of the three genes are indicated by boxes, with the intron in the GRP gene represented by a line. Start and stop codons are given. The number of nucleotides present in the 3′-noncoding and 5′-noncoding regions from the respective clones is given. Sequences derived from the genomic (gPR-1a, gGRP-8) or cDNA (pROB12) clones are indicated above the genes (number +31 in PR-S, Van Kan et al., 1988b). BamHI or HindIII restriction sites used for insertion of the coding sequences behind the CAMV 35S promoter are underlined.

Expression of PR-1a, GRP, and PR-S in Transgenic Plants

A total of nine regenerated kanamycin-resistant plants was obtained from the PR-1a transformation, 11 plants from the GRP transformation, and nine plants from the PR-S transformation. Total RNA was extracted from the transgenic plants and from uninfected and TMV-infected untransformed tobacco and analyzed on RNA gel blots. Figure 2 shows the results of hybridization of different PR cDNA probes to RNA preparations from several primary transformants. Hybridization with PR-1a cDNA probe (upper left panel) indicated specific expression of PR-1 in PR-1a transformants B36 and B39. PR-S transformant E110 shows a low endogenous expression of PR-1 mRNA. Lane T in each panel indicates the expression levels of the endogenous PR genes in infected plants, whereas lanes H show that nontransformed, noninduced tobacco contains no detectable PR messenger. When probing the different RNA preparations for the presence of a GRP messenger, a low level of endogenous expression became apparent in most transgenic and nontransformed plants. (Figure 2, upper right panel). However, the level of expression in GRP transformant C32 is greatly enhanced, suggestive of constitutive expression of the transgene. A probe for PR-Q indicates that the different transgenic plants do not express the endogenous acidic chitinase genes to any detectable level (Figure 2, lower left panel). Finally, in the lower right panel of Figure 2, hybridization to PR-S probe in the induced (lane T) and PR-S transgenic

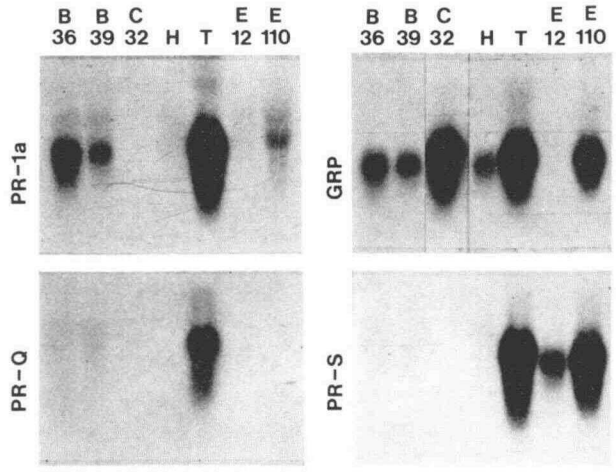

Figure 2. RNA Expression of Primary Transformants.

Four identical RNA gel blots containing total RNA extracted from uninfected (H) and TMV-infected (T) nontransformed tobacco and transgenic tobacco plants with chimeric genes for PR-1a (B36, B39), GRP (C32), and PR-S (E12, E110) were hybridized with cDNA probes for PR-1a, GRP, PR-Q, and PR-S (indicated to the left of each blot).

plants (E12 and E110) indicates the presence of PR-S RNA.

Figure 3 shows the results of RNA gel blot hybridizations with RNA preparations from second generation transformants. The top panel shows expression levels for PR-1a in B21 and B36 plants that are somewhat lower than those of the endogenous TMV-induced PR-1 genes (lane T). Plants E110 show a low level of endogenous PR-1 expression, as was the case with the primary transformant (Figure 2). The absence of hybridization in this and the other panels to the RNA preparation from noninduced tobacco (lane H) indicates the absence of expression of the endogenous PR genes. The second panel shows the high expression of GRP RNA in the GRP transgenic plants. The expression in plants C32 is markedly higher than in the TMV-induced plants. The third panel shows that PR-S plants E12 give low expression, whereas E110 plants give high expression of the PR-S gene. E19 plants, although kanamycin-resistant, do not show any detectable PR-S RNA.

Total leaf protein of the plants with high, specific RNA expression was analyzed on protein gel blots using polyclonal antisera for PR-1a and PR-S. The results are shown in Figure 4. The PR-1a antiserum cross-reacts with PR-1b and 1c. However, under the electrophoretic conditions used, PR-1a migrates more slowly than the other PR-1 proteins. This enables a positive determination of PR-1a expression. As can be seen in lanes B21 and B36, these transgenic lines specifically contain PR-1a, indicating expression of the transgene. Plants E12 and E19 also contain small amounts of PR-1 proteins. E12 expresses PR-1a, 1b, and 1c in the same ratio as the TMV-induced plants, whereas plants E19 seem to express only PR-1b and/or 1c. Plants E110 do not show a detectable level of PR-1, as is the case for the GRP transgenic plants (lanes C) and nontransformed, noninduced tobacco (lanes H). The bottom panel shows the presence of PR-S in induced (lanes T) and PR-S transgenic tobacco plants (E12 and E110). Plants E19 do not contain PR-S, which is in agreement with the absence of any detectable PR-S RNA (Figure 3). No antisera were available for the detection of GRP protein expression.

Infection of Transgenic Plants with TMV and AlMV

Plants from lines showing high, constitutive expression of the different chimeric genes were used for infection studies with TMV and AlMV. B21 and B36 plants were selected from the PR-1a transformants. GRP transformants were C11, C12, C15, C16, C21, and C32, whereas PR-S transformants were E12 and E110. Also tested were kanamycin-resistant plants B11, B31, C13, C14, and E19 from the PR-1a (B), GRP (C), and PR-S (E) transformations but with low or nondetectable expression of the chimeric genes. Nontransformed Samsun NN tobacco was used as a control.

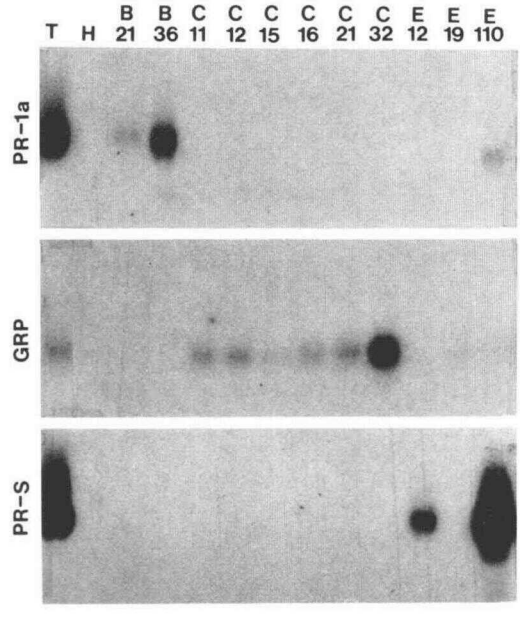

Figure 3. RNA Expression of Second Generation Transformants.

Three identical RNA gel blots containing total RNA isolated from uninfected (H) and TMV-infected (T) nontransformed tobacco and second generation transgenic tobacco with chimeric genes for PR-1a (B21, B36), GRP (C11, C12, C15, C16, C21, C32), and PR-S (E12, E19, E110) were hybridized with cDNA probes for PR-1a, GRP, and PR-S (indicated to the left of the panels).

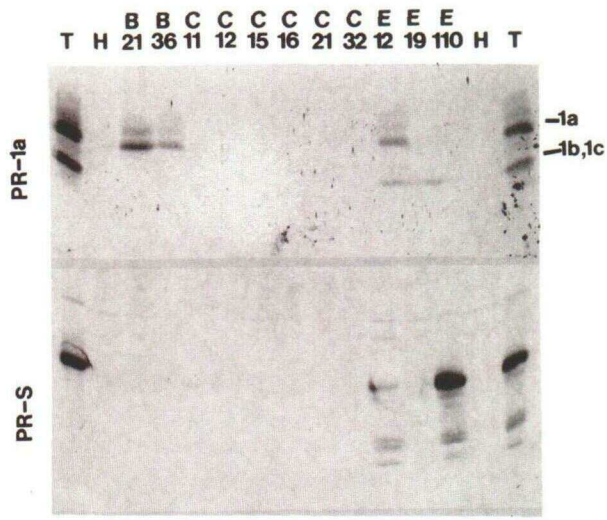

Figure 4. Protein Expression of Second Generation Transformants.

Protein gel blots containing protein extracts from plants of Figure 3 were incubated with antisera to PR-1a (top) and PR-S (bottom). Positions of PR-1a and 1b/1c are indicated to the right.

Upon inoculation with TMV, all plants became infected. Approximately equal numbers (30 to 100 per leaf) of local necrotic lesions appeared 2 to 3 days after inoculation that were indistinguishable in appearance from those of nontransformed plants. A representative example of the symptoms of TMV infection is shown in Figure 5. From incubation until 4 weeks postinoculation, some plants developed TMV symptoms in the noninoculated leaves and stem, indicating systemic spread of the infection. However, there was no correlation with the expression of a particular gene.

Possible effects of the constitutive expression of the different genes on the replication of a systemically spreading, non-necrotic virus were investigated by infection studies with AlMV. Infection became apparent 3 to 5 days after inoculation, when most plants began to show light chlorotic spots on the inoculated leaves, soon followed by chlorosis and formation of mosaic symptoms in the noninoculated leaves. Whereas most transgenic and nontransformed plants contained AlMV, as could be deduced from the local lesions on bean, some plants apparently did not contain large quantities of infectious virus, as can be seen in Table 1. Again, this did not correlate with the expression of a particular gene.

DISCUSSION

Transformation with constructs of PR-1a, GRP, and PR-S behind the CaMV 35S promoter leads to constitutive expression of these chimeric genes, as is shown by the presence of the respective transcripts in nonstressed tobacco (Figure 2). Van Kan et al. (1988a) have shown that the GRP gene that was used in these experiments is expressed in vivo. Hybrid selected in vitro translation products of RNA preparations from TMV-infected and salicylic acid treated Samsun NN tobacco reacted with an antiserum obtained against a synthetic C-terminal GRP peptide. However, this antiserum does not react to any protein in extracts from stressed tobacco, suggesting inaccessibility or posttranslational modification of GRP in the plant (Van Kan, 1988). Whatever reason, this inability to demonstrate the protein causes uncertainty about the messenger capability of the constitutively expressed GRP transcripts. A positive proof of the translational ability of the transcripts from the chimeric PR-la and PR-S genes was obtained by the reaction of the corresponding antisera to protein extracts from the transgenic plants (Figure 4).

One line of PR-S offspring (E19) did not show any detectable transgene expression, although kanamycin-re-

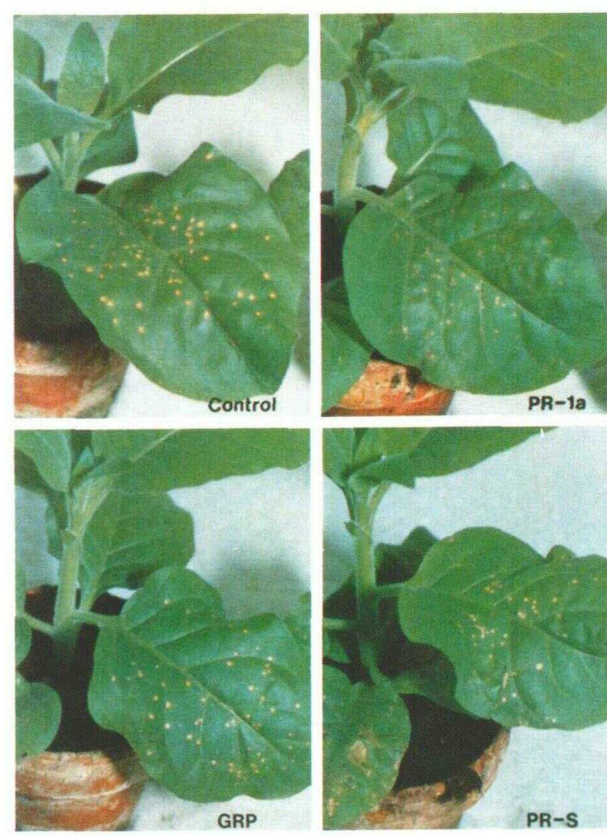

Figure 5. Infection of Transgenic Tobacco with TMV.

Symptoms of TMV infection on nontransformed (control) and transgenic tobacco plants expressing PR-1a (plant B21), GRP (plant C32), and PR-S (plant E12) as indicated.

Table 1. Infection of Transgenic Tobacco with AlMV[a]

Plant Line	Plant 1	2	3	4
1. B21	55	8	>300	>300
2. B36	155	>300	>300	>300
3. C11	30	69	124	172
4. C12	79	>300	52	63
5. C15	45	87	116	125
6. C16	64	49	230	25
7. C21	0	105	54	133
8. C32	0	54	>300	119
9. E12	>300	37	>300	197
10. E110	110	8	23	61
11. B11	0	0	247	225
12. B31	>300	85	>300	>300
13. C13	3	11	>300	197
14. C14	225	>300	139	71
15. E19	142	>300	108	121
16. Non[b]	197	65	90	225

[a] Values are average local lesion counts on duplicate bean half-leaves inoculated with diluted homogenates of AlMV-inoculated tobacco leaf from four plants of each transgenic line. B, C, and E lines are from PR-1a, GRP, and PR-S transformations, respectively. Tobacco lines 1 to 10 express the transgenes; lines 11 to 15 are kanamycin-resistant transformants not expressing the transgenes to detectable levels.

[b] Plants are nontransformed tobacco plants.

sistant and derived from a regenerated primary transformant, which constitutively expressed both PR-S RNA and protein to high levels (results not shown). All other selected lines had expression levels of the transgenes very similar to those of the primary transformants. Offspring of one PR-1a-expressing primary transformant (B39), after germinating from seed, did not develop roots, and was lost for further study. Constitutive expression of PR-1a cannot be the cause of this effect because two other PR-1a-expressing lines (B21 and B36) did not show any developmental abnormality. Neither does constitutive expression of GRP or PR-S influence the phenotypic appearance of tobacco to a large extent. Light variability in the growth rate of some lines was not obviously correlated to expression of a particular gene.

Expression of GRP does not induce expression of the stress-induced genes for PR-1, PR-S, or acidic chitinase (PR-P or PR-Q). Neither is this the case for constitutive expression of PR-1a. However, transgenic plants E110, which have expression levels for PR-S equal to or higher than that of TMV-induced tobacco, contained some PR-1 RNA (Figures 2 and 3), whereas PR-1 protein was present in PR-S transgenic plants E12 and E19. Studies with more independent transgenic lines have to confirm whether this apparent effect of PR-S on PR-1 expression is specific.

The results of infection experiments with TMV and AlMV indicate that the observed phenomenon of induced resistance to virus infection is not correlated directly with the presence of either PR-1a, GRP, or PR-S. However, resistance to virus infection in induced tobacco may be the result of the combined effect of several different PR or other induced proteins. An interspecific hybrid of *Nicotiana glutinosa* × *N. debneyi* that constitutively produces a PR-1 type protein was found to be highly resistant to infection with TMV and tobacco necrosis virus (Ahl and Gianninazzi, 1982). Our results indicate that it is not the PR-1 protein that is responsible for the resistance in this hybrid.

METHODS

Construction of Chimeric Genes and Transformation of Tobacco

The PR-1a sequence was derived from the genomic clone gPR-1a (clone 1 in Cornelissen et al., 1987). The promoter region and 5′-noncoding region were removed by Bal31 exonuclease treatment from the XhoI site approximately 900 bp in front of the transcription start site. This resulted in deletion of all nucleotides in front of the ATG start codon. After HindIII linker addition and digestion, the fragment containing the coding region and 322 3′-terminal base pairs was cloned into the unique HindIII site between the CaMV 35S promoter and nopaline synthase terminator of a transformation vector derived from pAGS 129 (Van Dun et al., 1987).

The coding sequence for GRP was derived from the genomic clone gGRP-8 (Van Kan et al., 1988a). A subclone in pUC 8 of a gGRP-8 HindIII fragment, containing 644 bp upstream and 157 bp downstream of the transcription start site, was digested with RsaI and EcoRI, and a fragment of approximately 200 bp was ligated into SmaI plus EcoRI-digested pIC-20H (Marsh et al., 1984). This resulted in the construction of a BamHI site 16 bp in front of the original transcription start site. This fragment was cleaved out of the vector with HindIII and PstI (which cleaves in front of the BamHI site in the polylinker of pIC-20H) and ligated to a 1121-bp-long HindIII-BamHI subfragment of gGRP-8 in a ligation mix containing BamHI plus PstI-digested pUC 8. This construction produced a BamHI fragment containing the coding sequence present on two exons and 5′-terminal and 3′-terminal noncoding regions, without the original TATA box. This BamHI fragment was cloned in the correct orientation behind the CaMV 35S promoter of the transformation vector pROK 1 (Baulcombe et al., 1986).

For the construction of the chimeric PR-S gene, a cDNA clone was used (pROB 12, Cornelissen et al., 1986) which contained the entire coding sequence. However, the three nucleotides present at the 5′ end in front of the ATG start codon were preceded by 13 guanosine residues derived from the oligo(G) tails of the vector. To avoid any influence of this G stretch in the translation of the transgenic messenger, a synthetic 23-bp-long restriction fragment with the 5′-terminal sequence of the cDNA clone, containing a BamHI site at one end and a HindIII site at the other end, was ligated onto the HindIII site present 18 bp from the 5′ end in pROB 12. Subsequently, digestion with BamHI and ligation of the resulting fragment into the BamHI site of pROK 1 (Baulcombe et al., 1986) resulted in a PR-S gene constructed behind the CaMV

35S promoter with a 3′-terminal noncoding sequence of approximately 190 bp, including an oligo(C/G) stretch and a pUC 9 polylinker region.

The correct sequence and orientation of all constructs behind the CaMV 35S promoter in the transformation vectors were verified by sequence analysis.

The above plasmids, all with the selectable neomycin phosphotransferase marker gene, were transferred from *Escherichia coli* to *Agrobacterium tumefaciens* strain LBA 4404 (Hoekema et al., 1983) by triparental mating using plasmid pRK 2013 in *E.coli* strain HB101 (Ditta et al., 1980). Tobacco (Samsun NN) leaf discs were infected with *A. tumefaciens* LBA 4404 containing the different transformation plasmids, and kanamycin-resistant plants were regenerated from transformed shoots as described by Horsch et al. (1985).

Analysis of Chimeric Gene Expression

Gene expression was measured by RNA and protein analysis of tobacco leaf extracts. Total RNA was analyzed on RNA gel blots (Sarachu et al., 1985) with ^{32}P-labeled probes derived from PR-1a, GRP, and PR-S cDNA clones (Hooft van Huijsduijnen et al., 1985) and a clone for the acidic chitinase PR-Q (L. C. Van Loon, unpublished results). Sap squeezed from young leaves was clarified by centrifugation. Equal amounts of protein were electrophoresed in polyacrylamide gels and blotted to nitrocellulose (Towbin et al., 1979). The protein gel blots were incubated with rabbit antisera raised against purified PR-1a and PR-S, after which alkaline phosphatase activity from the goat anti-rabbit conjugate indicated the presence of PR-1 and PR-S in the extracts.

Virus Infection Assays

Seed from selected transformed plants obtained after self-pollination was germinated (Van Dun et al., 1988) to assay second generation transformants for their susceptibility to virus infection. After dusting with carborundum powder, leaves of four plants of each line were inoculated mechanically with 50 μl of a solution containing 1 μg/ml TMV. The number of local lesions formed on the inoculated leaves quantitated the infectability of the transgenic plants with TMV. A leaf homogenate of an AlMV-infected tobacco plant was used to inoculate mechanically the three lower leaves of four plants of each transgenic line. After 10 days, homogenates (1 g in 1.6 ml of buffer containing 10mM Na-phosphate, pH 7, 1 mM EDTA) of inoculated leaf were diluted 1:200 and inoculated onto bean half-leaves. AlMV infection was measured by counting the number of local lesions on bean.

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