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Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group

Ben J.C.Cornelissen+, Jeannine Horowitz[1], Jan A.L.van Kan, Robert B.Goldberg[1] and John F.Bol*

Department of Biochemistry, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands and [1]Department of Biology, University of California, Los Angeles, CA 90024, USA

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ABSTRACT

Infection of Samsun NN tobacco with tobacco mosaic virus (TMV) was found to induce the synthesis of mRNA encoding a basic protein with a 67 % amino acid sequence homology to the known acidic pathogenesis-related (PR) proteins 1a, 1b and 1c. By Southern blot hybridization it was shown that the tobacco genome contains at least eight genes for acidic PR-1 proteins and a similar number of genes encoding the basic homologues. Clones corresponding to three of the genes for acidic PR-1 proteins were isolated from a genomic library of Samsun NN tobacco. The nucleotide sequence of these genes and their flanking sequences were determined. One clone was found to correspond to the PR-1a gene; the two other clones do not correspond to known TMV-induced PR-1 mRNA's and may represent silent genes. Compared to the PR-1a gene, these genes contain an insertion or deletion in the putative promoter region and mutations affecting the PR-1 reading frame.

INTRODUCTION

In response to infection by pathogens which produce local necrotic lesions, many plant species are known to accumulate pathogenesis-related (PR) proteins (1). These proteins have been studied in much detail because their synthesis is closely associated with the mechanism of induced resistance, i.e. the phenomenon that after a hypersensitive response the plant becomes resistant to further infection with the same or an unrelated pathogen (2). For example, in tobacco plants tobacco mosaic virus (TMV) induces resistance to viruses, bacteria and fungi; conversely, bacteria and fungi which produce necrotic lesions on tobacco induce resistance to TMV (see reference 3). At the molecular level, the PR proteins induced by TMV in Nicotiana tabacum cultivar Samsun NN (hereafter referred to as Samsun NN tobacco) have been characterized in most detail. Ten acidic proteins have been identified, designated PR-1a, 1b, 1c, 2, N, O, P, Q, R and S (4) which are all secreted by the plant cells into the intercellular space of the leaf (5). Serological studies have shown the existence of at least three groups of related proteins:

the PR-1 proteins (6), the proteins 2, N and O (7) and the proteins P and Q (8).

To further investigate the structure and function of these PR proteins, we have cloned DNA copies of six classes of TMV-induced mRNA's of Samsun NN tobacco, which were designated "cluster A to F" (9). Cluster B was found to correspond to the PR-1 proteins; a characterization of a full-length cDNA clone of PR-1b mRNA and incomplete cDNA clones of PR-1a and -1c mRNA's has been described (10). Cluster E encodes a PR protein which is homologous to the sweet-tasting protein thaumatin (11), and which has a amino acid composition similar to that of PR-R (12). Recently, clusters D and F were found to correspond to acidic and basic tobacco chitinases, respectively, the acidic chitinases being identical to PR proteins P and Q (8). As chitinases are potent inhibitors of fungal growth (13) these PR proteins may be involved in the anti-fungal response induced by TMV-infection. Little is known yet about the function of the proteins corresponding to clusters A and C. The mRNA's corresponding to clusters B and C are strongly induced by spraying tobacco plants with a solution of 5 mM salicylic acid. This treatment inhibits virus multiplication by a specific block of viral RNA synthesis without causing a detectable inhibition of host metabolism (14). Possibly, the proteins corresponding to clusters B and C are involved in the TMV-induced antiviral response.

In the present study we report that the genome of Samsun NN tobacco contains at least eight genes corresponding to acidic PR-1 proteins. Three of these genes were cloned and sequenced. In addition, it was found that the tobacco genome contains another family of genes encoding basic proteins with extensive sequence homology to the acidic PR-1 proteins.

MATERIALS AND METHODS

cDNA's

The cDNA clones used in this study have been described earlier (9,10).

Construction and screening of a tobacco genome library

Nuclear DNA isolated from young tobacco leaves (*Nicotiana tabacum* cv Samsun NN) according to Fischer and Goldberg (15) was cloned in the charon 32 vector (16) by the procedures of Zimmerman *et al*. (17). Approximately 2 x 10^6 independent isolates were plated and amplified to form a permanent library. The average tobacco DNA insert size was 18 kb. The library was screened for tobacco PR-1 genes using the plaque

hybridization procedure of Benton and Davis (18) and nick-translated (19) clone 69 DNA as probe.

Analysis of genomic clones

Recombinant phage DNA positively responding to the probe, was isolated according to Davis et al. (20) and Fischer (21), and analysed by restriction mapping and hybridization. Southern blot analysis (15) of EcoRI-HindIII double-digested DNA of the recombinant clones numbers 1, 2 and 12 revealed only one hybridizing fragment in each case. These fragments (a 3.3 kb EcoRI-HindIII fragment, a 2.2 kb EcoRI-HindIII fragment and a 2.3 kb HindIII fragment of number 1, 2 and 12, respectively) were subcloned into pUC9 and subsequently into M13 derivatives tg130 and tg131 (22). DNA was sequenced by the dideoxy chain terminating procedure of Sanger et al. (23) using [a-^{35}S] dATP (24).

Southern blot analysis of nuclear tobacco DNA

Nuclear DNA (10 mg) was digested with EcoRI or HindIII, electrophoresed on 1 % agarose gels, and transferred onto Gene-screen plus (New England Nuclear); subsequently, the blot was hybridized with ^{32}P-labeled cDNA (25). In reconstruction experiments amounts of recombinant phage DNA corresponding to one PR-1 gene copy in the nuclear DNA preparation was electrophoresed as well. The one kb DNA ladder (BRL) was used as molecular weight markers. The labeling of cDNA was done by nick-translation (19).

RESULTS

Characterization of PR-1 mRNA's

Previously, cDNA clones 119, 69 and 110 have been assigned to the PR 1a, -1b and -1c mRNA's, respectively, and sequencing of additional clones did not provide evidence for the existence of more than three PR-1 mRNA's (10). Only the PR-1b cDNA clone was full-length. Here, we report a further analysis of the cluster B cDNA clones isolated by Hooft van Huijsduijnen et al. (9). Sequencing PROB 35 showed it to be a nearly full-length clone encoding the signal peptide of 30 amino acids and a mature PR-1 protein of 138 amino acids. The sequence of the 3'-terminal half of this clone was found to be identical to that of the incomplete clone 119. The amino acid sequence encoded by PROB 35 shows four differences with the partial sequence of PR-1a that was determined at the protein level by Lucas et al. (26). However, because the available data indicate that there are only three PR-1 mRNA's and proteins and because

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                       20                    40                    60
                               **          *                       *
1a  QNSQQDYLDA HNTARADVGV EPLTWDDQVA AYAQNYASQL AADCNLVHSH GQYGENLAEG
1b  QNSQQDYLDA HNTARADVGV EPLTWDNGVA AYAQNYVSQL AADCNLVHSH GQYGENLAQG
G   QNSPQDYLNP HNAARRQVGV GPMTWDNRLA AFAQNYANQR AGDCRMQHSG GPYGENLAAA
       *    **   *  **    * *    **   *    ** *  *  ***  *  *      **

                       80                   100                   120
                                                              *
1a  SGDFMTAAKA VEMWVDEKQY YDHDSNTCAQ GQVCGHYTQV VWRNSVRVGC ARVQCNNGGY
1b  SGDFMTAAKA VEMWVDEKQY YDHDSNTCAQ GQVCGHYTQV VWRNSVRVGC ARVKCNNGGY
G   YPQPH-AAGA VKMWVDEKQF YNYNSNTCAA GNVCGHYTQV VWRNSVRLGC ARVRCNNGWY
    ******  *   *       *  ***     *  *                *      *    *

                      140                   160
                ** *
1a  VVSCNYDPPG NYRGESPY
1b  VVSCNYDPPG NVIGQSPY
G   FITCNYDPPG NWRGQRPTVI LKSNIPLIPS WNFQLMSSNN GLRDQIMNKS FVMC
    ***         **  * *
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Fig. 1 Comparison of the amino acid sequences of PR-1a, PR-1b and the protein encoded by the cluster G clone PROB 51. Amino acids in 1a and G that differ from the 1b sequence are indicated by asterisks. The N-terminal signal sequences are not included. The PR-1b sequence is taken from (10).

the proteins encoded by clones 69 and 110 show considerably more than four differences with the sequence reported by Lucas et al. (26), we feel that it is justified to assume that PROB 35 corresponds to PR-1a mRNA. Figure 1 shows that there are eight differences between the amino acid sequences of PR-1a and -1b, i.e. they are 94 % homologous. Cluster B was found to contain two clones, PROB 24 and PROB 51, which cross-hybridized to PR-1b cDNA only when the hybridization was done at low stringency. The two clones, with inserts of different lengths, corresponded to the same mRNA. The insert in PROB 51 was nearly full-lenght: it lacked the sequence corresponding to the 5′-non-coding region and part of the region encoding the signal peptide. For reasons given below PROBs 24 and 51 are considered to represent a new class of TMV-inducible mRNA's, designated cluster G. The length of the PROB 51 encoded mature protein is 173 amino acids. Figure 1 shows that there is a 67 % homology between the N-terminal sequence of 137 amino acids of this protein and the PR-1b sequence. The C-terminal extension with 36 amino acids of the cluster G protein is due to the fact that the TAA termination codon in PR-1a and -1b cDNA's is changed into GTG in PROB's 24 and 51.

A comparison of PR-1b and the G-protein shows that many acidic and neutral residues in PR-1b are replaced by neutral and basic residues, respectively, in the G-protein. Table 1 lists the amino acid composition of PR-1a, -1b, the G-protein and p14, a PR-1 type protein induced by TMV-

Table I. Amino acid composition of proteins from the PR-1 group

	1a	1b	G	p14
Ala (A)	15	14	16	14
Val (V)	14	16	11	9
Leu (L)	5	5	8	6
Ile (I)	0	1	5	3
Pro (P)	4	4	11	5
Phe (F)	1	1	5	2
Trp (W)	3	3	6	5
Tyr (Y)	11	10	9	7
Met (M)	2	2	6	1
Gly (G)	12	13	14	16
Ser (S)	8	8	9	8
Thr (T)	5	5	5	3
Cys (C)	6	6	7	6
Asn (N)	11	12	21	13
Gln (Q)	12	12	12	8
Asp (D)	11	10	6	6
Glu (E)	6	4	2	2
Lys (K)	2	3	4	3
Arg (R)	5	4	12	10
His (H)	5	5	4	3
	138	138	173	130

Amino acid composition of PR-1b is taken form Cornelissen et al. (10); the composition of the tomato protein p14 is from Lucas et al. (26).

or viroid-infection of tomato (26). PR-1a and -1b contain an excess of acidic amino acids in agreement with their isoelectric point of 4.0 (27). The G-protein contains an excess of basic residues like the p14 protein which has an isoelectric point of 10.7 (28). The sequence homolgy between p14 and the N-terminal 137 amino acids of the G-protein is 61 % (result not shown); a similar homology has been reported for p14 and PR-1b (10).

Characterization of PR-1 genes

To determine the number of PR-1 genes in the Samsun NN genome, nuclear DNA was analyzed by the Southern blot technique. In the left panel of Figure 2 tobacco DNA digested with HindIII (lane 1) or EcoRI (lane 2) was probed with PR-1b cDNA (clone 69). A shorter exposure of the blot revealed that the 10.0 and 8.0 kb bands in lane 1 and the 6.0 and 3.5 kb bands in lane 2 were doublets. In view of its intensity, the 2.2 kb band in lane 1 is probably also a doublet. This indicates that with each of the two restriction enzymes eight fragments of nuclear DNA are generated which contain PR-1 specific sequences. To analyze these fragments in more detail, a genomic library of Samsun NN tobacco was

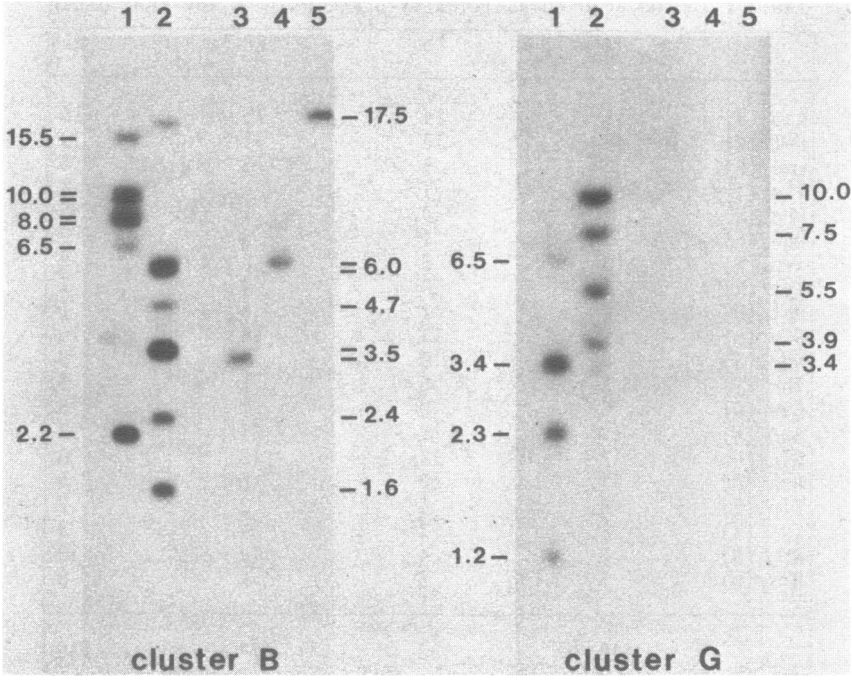

Fig. 2 Southern blot analysis of genes encoding acidic PR-1 proteins (Cluster B) and basic PR-1 like proteins (Cluster G). Lanes 1 and 2 were loaded with nuclear DNA of Samsun NN tobacco digested with HindIII and EcoRI, respectively; lanes 3, 4 and 5 were loaded with EcoRI digests of the genomic clones 1, 2 and 12, respectively. The blots were hybridized to ^{32}P-labeled cDNA clone 69 (left panel) or PROB 24 (right panel). The estimated size of the fragments (kb) is indicated.

screened with PR-1b cDNA as probe. Three positively responding clones, numbers 1, 2, and 12, were found to have unique restriction patterns. In lanes 3, 4, and 5 of Figure 2, EcoRI digests of these clones were loaded in amounts equivalent to one PR-1 gene copy in the nuclear DNA digests that were loaded in lanes 1 and 2. A single EcoRI fragment of each genomic clone was found to hybridize with PR-1b cDNA (Figure 2, left panel). These fragments comigrated with EcoRI fragments in the digest of nuclear DNA shown in lane 2. As will be shown below clones 1, 2, and 12 each contain one gene homologous to the PR-1b probe. In view of the intensity of the bands in the digests of nuclear DNA, it may be concluded that the eight bands seen in lanes 1 and 2 of Figure 2 (left panel)

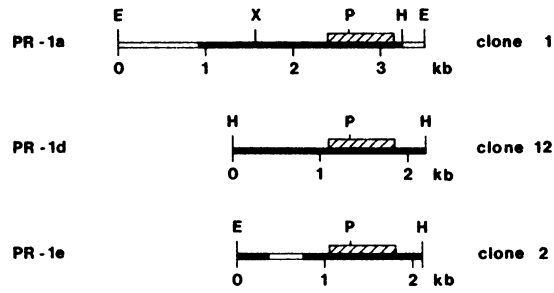

Fig. 3 Schematic representation of the restriction fragments of genomic clones 1, 2 and 12 that were subcloned for sequence studies. The regions indicated by solid bars have been sequenced. The hatched boxes show the location of the PR-1 genes. The size of the fragments (kb) is indicated. E, EcoRI; H, HindIII; P, PstI; X, XhoI.

correspond to at least eight genes for acidic PR-1 proteins in the Samsun NN genome.

In the right panel of Figure 2 the blot was hybridized to the cDNA clone PROB 24 representing cluster G. Under the conditions used, there was no cross-hybridization with the genomic PR-1 clones loaded in lanes 3, 4, and 5. Four to five bands in lanes 1 and 2 do hybridize to the cluster G probe. These bands differ in migration rate from the bands hybridizing to the cluster B probe. The data do not allow the determination of the exact number of cluster G genes. In view of the unequal intensity of the bands it may well be that there are also eight genes for basic PR-1 like proteins in the Samsun NN genome.

Structure of PR-1 genes

Figure 3 shows the restriction fragments of genomic clones 1, 2 and 12 that were subcloned in M13 derived vectors. The regions indicated by the solid bars were sequenced; the hatched box shows the location of the PR-1 gene in each clone. The genes do not contain introns. The sequence of the gene in clone 1 was identical to that of the PR-1a cDNA clone (PROB 35). The genes in clones 2 and 12 were approximately 90 % homologous though not identical to cDNA of PR-1a, -1b or -1c. Probably, clones 2 and 12 correspond to PR-1 genes that are not expressed after TMV-infection of tobacco. These genes are designated PR-1d and -1e in Figure 3.

Figure 4 shows the complete sequence of the PR-1a gene and its

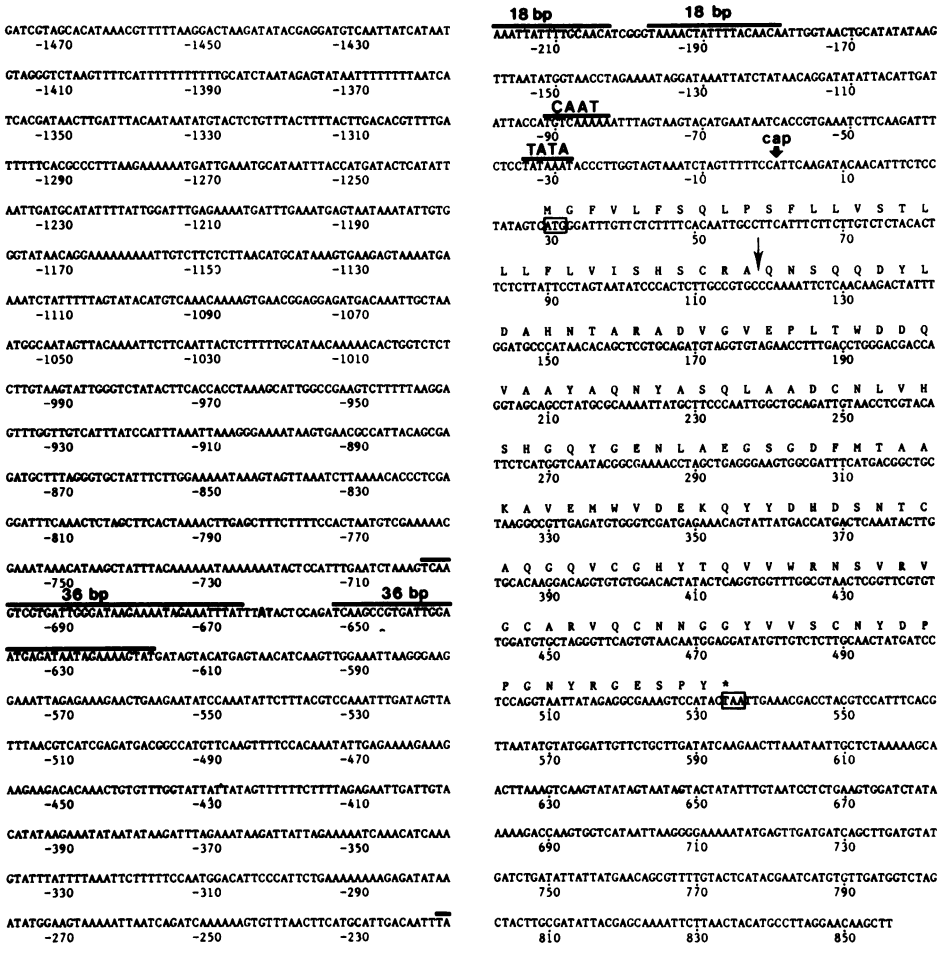

Fig.4 Nucleotide sequence of the PR-1a gene and its flanking sequences. The PR-1a reading frame is aligned with the corresponding amino acid sequence; initiation and termination codons are boxed. The cap site is indicated with a bold arrow; a thin arrow indicates the cleavage site involved in removal of the signal peptide (29). The cDNA clones terminate around position 776. Putative TATA - and CAAT - boxes and the location of imperfect repeats of 18 and 36 bp are indicated.

flanking regions. The position of the cap-site has been determined previously (29); the cDNA clones terminate around position 776 down stream this cap-site but no clear polyadenylation signal is present in the 3'-non-coding sequence (10). Upstream of the cap-site putative TATA - and CAAT - boxes are found at the expected positions. In addition,

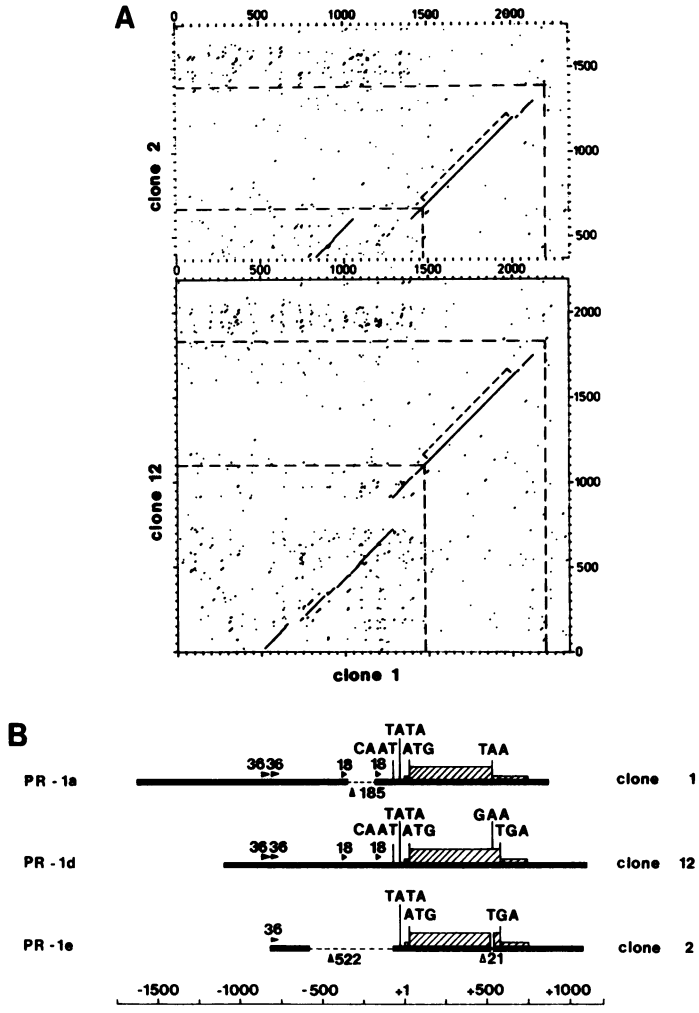

Fig. 5 Panel A. Dot-plot comparison of the sequence of genomic clone 1 with those of clones 2 and 12. Nucleotide numbers are given along the axes. The sequences were compared with a window of 21 nucleotides and a stringency of 14 nucleotides. The PR-1 genes in the three sequences are located between the broken lines; the position of the PR-1 reading frame is indicated along the diagonal. Panel B. Schematic representation of the sequence of clones 1, 2 and 12. Deletions (Δ) in clones 1 and 2 relative to the clone 12 sequence are indicated by dotted lines. The scale is in base pairs.

imperfect repeats of 18 and 36 bp are indicated in Figure 4.

Interestingly, just upstream of the putative TATA-box a sequence (nucleotides -57 to -43) is found which strongly resembles the heat shock

consensus sequence 5' CT-GAA--TTC-AG 3' (30). Synthesis of PR-1 proteins, however, is not induced by heat-shock treatment (R.A.M. Hooft van Huijsduijnen and J.F. Bol, unpublished results), thus the significance of this sequence homology is doubtful.

The sequences of clones 1, 2 and 12 were compared to each other by making "dot plots" using a UWCG computer program. In Figure 5A the sequence of clone 1 is plotted against the sequences of clones 12 and 2; the sequences were compared using a window of 21 nucleotides and a stringency of 14 nucleotides. The PR-1 gene in each clone is located between the broken lines. The diagonal line between these broken lines illustrates the homology between the PR-1 genes; the diagonal line in the lower left parts of Figure 5A demonstrates that there is also considerable homology in the A/T-rich sequences upstream of the PR-1 genes. The discontinuities in this part of the diagonal reflect deletions/insertions in the putative promoter region. Little homology was observed between the sequences downstream the three PR-1 genes.

A schematic representation of the PR-1 genes is shown in figure 5B. Compared to the clone 1 sequence, the clone 12 sequence contains an insertion of 185 bp between the 18 bp repeats. Possibly, this insertion affects the expression of the gene. Moreover, the TAA termination codon of the PR-1a reading frame is changed into GAA in the PR-1d sequence giving an extension of the reading frame with 16 triplets until a TGA-triplet is encountered. Compared to the clone 12 sequence the clone 2 sequence contains a deletion of 522 bp in the putative promoter region of the PR-1e gene and a deletion of 21 bp around the position corresponding to the TAA termination codon of the PR-1a gene. These modifications corroborate the notion that the PR-1d and -1e genes are silent genes.

DISCUSSION

In the present study we have shown that the genome of Samsun NN tobacco contains a minimum of eight genes encoding acidic PR-1 proteins and a possibly similar number of genes encoding basic equivalents of these proteins. Probably, only three of the genes for the acidic proteins and at least one gene for a basic protein is expressed after TMV-infection. Nicotiana tabaccum cv Samsun NN is amphidiploid, containing 2n chromosomes from Nicotiana sylvestris and 2n chromosomes from Nicotiana tomentosiformis. It may well be that one part of the cluster B and G genes is derived from one parent, the other part being derived from the

other parent. Genetic studies with Samsun NN tobacco and its parents have shown that the genes for the acidic proteins PR-1a and -1c originate from N. Sylvestris, whereas the PR-1b gene is derived from N. tomentosiformis (31). As the isolation of PR proteins from tobacco has been directed towards the purification of acidic proteins and these proteins were mainly analyzed in alkaline non-denaturing gels (4, 32), the basic counterparts of the known PR-1 proteins probably have escaped detection so far. As the acidic tobacco PR-1 proteins are serologically related to the basic p14 protein from tomato (33), antisera to these PR-1 proteins can probably be used to detect the basic PR-1 like proteins in tobacco.

Recently, the ~ 25 kd PR proteins P and Q corresponding to cluster D and the ~ 34 kd proteins corresponding to cluster F were found to be acidic and basic chitinases, respectively, sharing a 65 % amino acid sequence homology (8). The physiological meaning of the occurrence of acidic and basic forms of these hydrolytic enzymes with putative anti fungal activity is not yet known. The finding that also the acidic PR-1 proteins have basic counterparts may suggest that these proteins have an enzymatic function too, despite the observation that these are not isozymes of 25 different enzymes known to increase in activity following TMV infection (1).

PR proteins have been detected in at least 16 plant species and serologically related PR-1 type proteins are produced in both monocotyledonous and dicotyledonous plants including maize, barley, Gomphrena globosa, Chenopodium amaranticolor, tobacco, tomato and potato (34). In most of these plants the PR-1 type proteins were detectable only after infection with pathogens or treatment with salicylic acid. These data indicate that the PR-1 genes are highly conserved in the plant kingdom and are induced in a similar way in a wide range of plants. The cloning and sequencing of PR-1 genes reported in this study sheds light on the structure of these genes and permits an analysis of the regulatory elements involved in their induction. The identification of possible nuclear proteins binding to these regulatory sequences may provide further insight in the sequence of events that results in the expression of these genes.

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*To whom correspondence should be addressed
+Present address: MOGEN International B.V., Einsteinweg 97, 2333 CB Leiden, The Netherlands

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