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DNA sequence analysis of a *PR-1a* gene from tobacco: Molecular relationship of heat shock and pathogen responses in plants

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Summary. Pathogenesis-related proteins (PR-proteins) are a heterogeneous group of host encoded, low molecular weight proteins, which are induced in plants by different external stimuli such as pathogen attack or exposure of the plants to certain chemicals. Three independent bacteriophage λ clones hybridizing to *PR-1* cDNA probes were isolated from a tobacco (*Nicotiana tabacum* cv. Wisconsin 38) genomic library. All three clones are derived from different genomic locations and contain single gene copies. DNA sequence analysis revealed that one of the genomic clones is essentially identical to the *PR-1a* cDNA clones described previously from tobacco mosaic virus infected tobacco plants (*Nicotiana tabacum* cv. Samsun NN). This clone contains 335 bp of the 5′ upstream and the entire transcribed regions. The *PR-1a* gene is not interrupted by intervening sequences. In the 5′ upstream region, characteristic eukaryotic transcription signals as well as putative regulatory sequences were identified. A consensus TATA box is found at position −34 and an 11 bp long imperfect direct repeat is present at positions −116 and −135 with respect to the transcription initiation site. Furthermore, the sequence C−−GAA−−−TTC−−G, which differs from the consensus heat shock regulatory element only by the insertion of one extra nucleotide, is located at position −57. The presence of this element suggests a molecular relationship of heat shock and pathogen responses in plants.

Key words: Heat shock regulatory element – Pathogenesis-related proteins (PR-proteins) – Plant gene sequence

Introduction

Infection of a variety of different plant species with pathogens leads to the de novo synthesis of several host encoded, low molecular weight proteins, known as pathogenesis-related proteins (PR-proteins; for review see van Loon 1985). The best characterized of these are the tobacco *PR-1* proteins. This group consists of three closely related members, *PR-1a, PR-1b*, and *PR-1c*, which have recently been characterized in detail by cDNA cloning (Cornelissen et al. 1986; Pfitzner and Goodman 1987). Hybridization of cDNA probes to genomic DNA blots strongly suggested that these proteins are encoded by a small multigene family containing at least eight members (Pfitzner and Goodman 1987).

PR-1 proteins were first described in tobacco cultivars exhibiting the hypersensitive response after tobacco mosaic virus (TMV) infection (Gianinazzi et al. 1970; van Loon and van Kammen 1970). Meanwhile, however, it has been shown that these proteins can accumulate under various alternate environmental conditions. They were found in tobacco plants at the onset of flowering (Fraser 1981) as well as in tobacco callus cultures (Antoniw et al. 1981). In addition, *PR-1* proteins can be induced artificially in healthy tobacco plants by the application of chemicals like acetylsalicylic acid, polyacrylic acid, or ethylene (White 1979; Gianinazzi and Kassanis 1974; van Loon 1977). The increase in *PR-1* proteins in tobacco plants infected with TMV and in plants exposed to acetylsalicylic acid is the result of highly elevated steady state mRNA levels (Cornelissen et al. 1986; Pfitzner and Goodman 1987; Carr et al. 1985). Moreover, it has been demonstrated that treatment of cell suspension cultures with elicitor results in a marked transcriptional activation of PR-protein genes in parsley (Somssich et al. 1986), thus suggesting that *cis*-acting regulatory sequences might govern their expression. To understand the regulation of PR-protein genes by different external stimuli and to investigate whether such regulatory sequences are associated with these genes, we have screened a genomic library for *PR-1* protein containing clones.

Here, we report the isolation and characterization of one gene encoding the tobacco *PR-1a* protein. Comparison of the genomic to the cDNA clones reveals that *PR-1* genes are not interrupted by intervening sequences. Furthermore, an 11 bp long imperfect direct repeat and a sequence highly homologous to the consensus heat shock regulatory element (HSE) have been identified in the 5′ upstream region of the *PR-1a* gene.

Materials and methods

Plant material. Tobacco plants were grown under normal greenhouse conditions at 20° C. When 2–3 months old, the plants were mock inoculated or infected with tobacco mosaic virus as described (Pfitzner and Goodman 1987) and harvested 7 days later.

RNA and DNA isolation. RNA was extracted from tobacco leaves as described (Pfitzner and Goodman 1987). Poly(A^+) RNA was selected by oligo(dT)-cellulose chromatography

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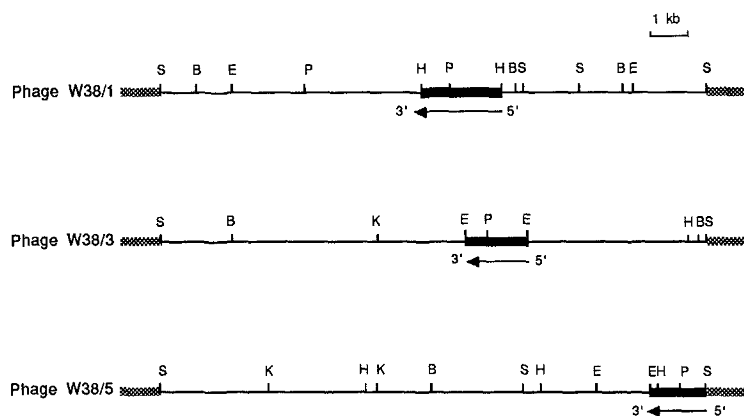

Fig. 1. Partial restriction maps of three independent *PR-1* genomic clones. The approximate locations of the *PR-1* genes within the clones as determined by hybridization with a *PR-1a* cDNA probe is shown by the *solid boxes* and the direction of transcription by the *arrows*. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I

(Aviv and Leder 1972). Plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly 1979).

Screening of the genomic library. About 10^6 recombinant plaques of a tobacco (*Nicotiana tabacum* cv. Wisconsin 38) genomic library in the bacteriophage λ vector EMBL3, kindly provided by Mark A. Conkling, were screened using nick translated pNt^{SNN}cPR1a/35 probe (specific activity 5×10^8 cpm/µg; Pfitzner and Goodman 1987). Filter hybridizations were performed according to standard procedures (Maniatis et al. 1982), and positive plaques were purified twice. DNA of recombinant clones was prepared from liquid cultures grown on *Escherichia coli* K12 strain LE392 and analyzed by digestion with restriction enzymes.

DNA sequence analysis. Nucleotide sequences were determined using the dideoxynucleotide chain termination method (Sanger et al. 1977) with α-[^{35}S]dATP (Biggin et al. 1983) and the universal external M13 primer. Restriction enzyme fragments were subcloned into M13mp18 and mp19 vectors (Yanisch-Perron et al. 1985) prior to sequencing.

Primer extension analysis. A synthetic 30 nucleotide long oligodeoxynucleotide complementary to positions 108 to 137 in the sequence (Fig. 2) was end-labeled with T4 polynucleotide kinase and γ-[^{32}P]ATP (specific activity 8×10^8 cpm/µg). About 7.5 ng primer were annealed to 0.5 µg poly(A$^+$) RNA from either mock inoculated or TMV infected tobacco plants in a 15 µl reaction containing 100 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 140 mM KCl, 20 mM β-mercaptoethanol, 1 mM dNTPs, and 40 units RNasin (Promega Biotech). The reaction was carried out at 42° C for 45 min in the presence of 21 units AMV reverse transcriptase (Life Sciences). The primer extension products were analyzed on a 6% polyacrylamide-8M urea sequencing gel along with the products of a dideoxy sequencing reaction generated by using the same primer and an M13mp18 template containing the 0.6 kb *Sal*I/*Pst*I fragment of the *PR-1a* gene.

Results

Isolation and characterization of genomic clones encoding PR-1 proteins

A tobacco (*N. tabacum* cv. Wisconsin 38) genomic library constructed in the bacteriophage λ vector EMBL3 (Conkling and Goodman, unpublished results) was screened by plaque hybridization using the cDNA clone pNt^{SNN}cPR1a/35 (Pfitzner and Goodman 1987). Out of 10^6 recombinant phages, 4 *PR-1* specific clones were obtained. Analysis of these clones by digestion with various restriction endonucleases and hybridization of the resulting fragments with the cDNA probe indicated that the clones are derived from 3 different genomic loci (Fig. 1). Each clone contains only a single gene copy. The approximate locations of the *PR-1* genes are confined to a 2.3 kb *Hin*dIII fragment in λ W38/1, to a 2 kb *Eco*RI fragment in λ W38/3, and to a 1.4 kb *Sal*I/*Eco*RI fragment in λ W38/5 (Fig. 1). These fragments were subcloned into pUC18 or pUC19 vectors. For further analysis, the clones were digested with restriction endonucleases specific for the 3′ untranslated regions of cDNA clones encoding *PR-1a, PR-1b,* and *PR-1c* proteins, respectively, and hybridized with the 340 bp *Pst*I/*Eco*RV fragment of pNt^{SNN}cPR1a/35. One of the genomic clones, λ W38/5, gave rise to a *Pst*I/*Eco*RV fragment identical in size to the corresponding fragment obtained from the cDNA clone pNt^{SNN}cPR1a/35 (data not shown). This result, therefore, suggested that λ W38/5 encodes the *PR-1a* protein and that intervening sequences are not present in this region of the gene.

Primary structure of the PR-1a *gene*

The genomic nucleotide sequence of the 1.4 kb *Sal*I/*Eco*RI fragment of λ W38/5 is shown in Fig. 2. The DNA sequence of the gene is identical to the previously isolated cDNA clones pNt^{SNN}cPR1a/8 and pNt^{SNN}cPR1a/35 encoding the *PR-1a* protein from *N. tabacum* cv. Samsun NN apart from three nucleotide changes. Two of these substitutions are located in the coding region and give rise to amino acid changes. These are a G→A (Asp106→Asn106) at position 345 and a T→A (Ser153→Thr153) at position 486. The third change, T→C, is located at position 601 in the 3′ untranslated region of the gene. Several lines of evidence indicate that these differences represent slight variations in the primary structures of *PR-1a* proteins from different tobacco cultivars: (1) when fused to a reporter gene, the genomic flanking sequence of the *PR-1a* gene is functional as a promoter element in transient expression studies (A.J.P. Pfitzner, U.M. Pfitzner and H.M. Goodman, unpublished results), (2) the other two genomic clones, λ W38/1 and λ W38/3, are clearly different in their DNA sequences from any cDNA clone previously characterized and are most probably pseudogenes (U.M. Pfitzner, A.J.P. Pfitzner and H.M. Goodman, unpublished results), (3) one of the amino

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- 335 GATCGTTAAATGTAGAAAATATTAATTAACACATTAACCATAACCAGTCTACTTTATTTAACAAAAAGCACATCTGATAG

- 255 ATCAAAAAAGTGTTTAACTTCATGCATTGACAATTTAAAATTATTTTGCAACATCGGGTAAAACTATTTTACAACAATTG
                                                          →                    →
- 175 GTAACTGCATATATAAGTTTAATATGGTAACCTAGAAAATAGGATAAATTATCTATAACAGGATATATTACATTGATATT

                                   H S E              →         ←
                                 *  ***  -**   -
-  95 ACCATGTCAAAAAATTTAGTAAGTACATGAATAATCACCGTGAAATCTTCAAGATTTCTCCTATAAATACCCTTGGTAGT
                                               *  ***   ***  *
              ↓+1                              HSE-like motif
-  15 AAATCTAGTTTTTCCATTCAAGATACAACATTTCTCCTATAGTCATGGGATTTGTTCTCTTTTCACAATTGCCTTCATTT
                                                       ↑
                                                   M  G  F  V  L  F  S  Q  L  P  S  F

   66 CTTCTTGTCTCTACACTTCTCTTATTCCTAGTAATATCCCACTCTTGCCGTGCCCAAAATTCTCAACAAGACTATTTGGA

       L  L  V  S  T  L  L  L  F  L  V  I  S  H  S  C  R  A  Q  N  S  Q  Q  D  Y  L  D

  146 TGCCCATAACACAGCTCGTGCAGATGTAGGTGTAGAACCTTTGACCTGGGACGACCAGGTAGCAGCCTATGCGCAAAATT

        A  H  N  T  A  R  A  D  V  G  V  E  P  L  T  W  D  D  Q  V  A  A  Y  A  Q  N  Y

  226 ATGCTTCCCAATTGGCTGCAGATTGTAACCTCGTACATTCTCATGGTCAATACGGCGAAAACCTAGCTGAGGGAAGTGGC

         A  S  Q  L  A  A  D  C  N  L  V  H  S  H  G  Q  Y  G  E  N  L  A  E  G  S  G

  306 GATTTCATGACGGCTGCTAAGGCCGTTGAGATGTGGGTCAATGAGAAACAGTATTATGACCATGACTCAAATACTTGTGC
                                              |
                                              G(→D)
       D  F  M  T  A  A  K  A  V  E  M  W  V  N  E  K  Q  Y  Y  D  H  D  S  N  T  C  A

  386 ACAAGGACAGGTGTGTGGACACTATACTCAGGTGGTTTGGCGTAACTCGGTTCGTGTTGGATGTGCTAGGGTTCAGTGTA

        Q  G  Q  V  C  G  H  Y  T  Q  V  V  W  R  N  S  V  R  V  G  C  A  R  V  Q  C  N

  466 ACAATGGAGGATATGTTGTCACTTGCAACTATGATCCTCCAGGTAATTATAGAGGCGAAAGTCCATACTAATTGAAACGA
                          |
                          T(→S)
         N  G  G  Y  V  V  T  C  N  Y  D  P  P  G  N  Y  R  G  E  S  P  Y  *

  546 CCTACGTCCATTTCACGTTAATATGTATGGATTGTTCTGCTTGATATCAAGAACTCAAATAATTGCTCTAAAAAGCAACT
                                                                  |
                                                                  T
  626 TAAAGTCAAGTATATAGTAATAGTACTATATTTGTAATCCTCTGAAGTGGATCTATAAAAAGACCAAGTGGTCATAATTA

  706 AGGGGAAAAATATGAGTTGATGATCAGCTTGATGTATGATCTGATATTATTATGAACACTTTTGTACTCATACGAATCAT
                                 ↑A6
  786 GTGTTGATGGTCTAGCTACTTGCGATATTACGAGCAAAATTCTTAACTACATGCCTTAGGAACAAGCTTACACAGTTCAT
                             ↑A48
  866 ATAATCTACTAGAGGGCCAAAAACATGAAAATTACCAATTTAGATGGTAGGAGGATATTGAAAGTGGAGCAGCTAGTTTT

  946 AATAACTGACCGTTAGTCTTAAAATTGACGGTATAAAAATATTTACATAATCAGGTCATTTATAAGGTAATTATAGGTAA

 1026 ATA
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Fig. 2. Nucleotide sequence and deduced amino acid sequence of the *PR-1a* gene and 5′ and 3′ flanking regions. The sequence of the coding strand is shown numbered in the left-hand column and by a dot above every tenth nucleotide. The nucleotide sequence of the genomic clone is compared to that of two cDNA clones published previously (Pfitzner and Goodman 1987). Identical nucleotides are indicated by *blanks*, differences by the changed nucleotide and amino acid substitutions are given next to the changed nucleotide in parentheses. The cDNA sequence starts at position 35 marked by the *vertical arrow.* Two in vivo polyadenylation sites at positions 732 and 808 are indicated. The major start of transcription, A, is labeled ↓+1. Important features described in the text are marked as follows: The consensus TATA box at position −34 and an inverted match to the CCAAT sequence at position −179 are shown in *bold letters.* Repeat elements are *overlined* and their relative orientation is given by *horizontal arrows.* The heat shock element (HSE)-like motif at position −57 is indicated. Matches to the consensus HSE are denoted by (*) and mismatches by (−)

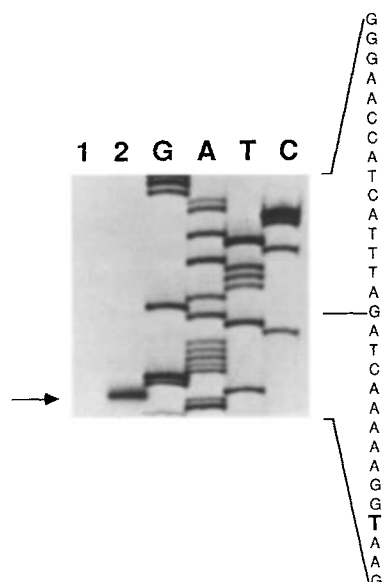

Fig. 3. Primer extension analysis of tobacco *PR-1* genes. A synthetic oligodeoxynucleotide homologous to *PR-1* genes was used to prime synthesis on 0.5 µg poly(A^+) RNA from mock inoculated tobacco plants (lane 1) and from tobacco mosaic virus infected plants (lane 2). The *arrow* indicates the major mRNA reverse transcription product observed (lane 2). The sequencing ladder (lanes GATC) was generated with the same oligodeoxynucleotide primer and an M13mp18 template containing the 0.6 kb *SalI*/*PstI* fragment of the *PR-1a* gene. The sequence of the noncoding strand shown is indicated next to the gel. Only the relevant region of the gel is shown; no other bands were seen elsewhere in the gel.

acid changes, Ser153→Thr153, is a conservative one, (4) the other amino acid substitution, Asp106→Asn106, is observed at the same position in the cDNA clones encoding the *PR-1c* protein (Pfitzner and Goodman 1987), (5) genomic Southern blots with DNA from Samsun NN and Wisconsin 38 tobacco yield nearly identical hybridization patterns, suggesting that both cultivars contain an equal number of highly homologous *PR-1* genes (data not shown), and (6) treatment of Samsun NN as well as Wisconsin 38 tobacco with acetylsalicylic acid leads to the induction of *PR-1* proteins displaying similar electrophoretic mobilities on two-dimensional polyacrylamide gels (data not shown). Thus, clone λ W38/5 most presumably contains an active gene encoding the *PR-1a* protein from *N. tabacum* cv. Wisconsin 38.

The genomic sequence contains an uninterrupted open reading frame of 504 nucleotides. The primary structure of the *PR-1a* protein is homologous to the *PR-1b* and *PR-1c* proteins, including a 30 amino acid signal peptide typical of eukaryotic exoproteins and a mature protein of 138 amino acids in length (Cornelissen et al. 1986; Pfitzner and Goodman 1987). *PR-1* genes do not contain introns. At the 3′ end of the *PR-1a* gene, multiple polyadenylation sites have previously been identified resulting in 194 bp and 270 bp long 3′ noncoding regions (Pfitzner and Goodman 1987). The DNA site corresponding to the 5′ ends of *PR-1* mRNAs was determined by primer extension experiments. Only one major extension product was observed using poly(A^+) RNA from TMV infected leaves (Fig. 3). The position of the transcription initiation site defined by this experiment is identicial to the 5′ ends of cDNA clones encoding the *PR-1b* and *PR-1c* proteins characterized previously (Cornelissen et al. 1986). Thus, the 5′ untranslated leader regions are presumably of an equal length (29 bp) for all *PR-1* genes.

5′-Nucleotide sequence of the PR-1a *gene*

The 335 bp genomic 5′ flanking sequence of the *PR-1a* gene contained in clone λ W38/5 was determined by DNA sequence analysis. Visual inspection of this region led to the identification of structural elements, which might be involved in the regulation of *PR-1* gene expression by environmental stimuli. At position −34 with respect to the start of transcription (+1, Fig. 2), a consensus TATA box and at position −179 an inverted match to the CCAAT sequence were found (Breathnach and Chambon 1981). Furthermore, an imperfect direct repeat of 11 bp is present at positions −116 and −135, and an 8 bp inverted repeat is located at positions −45 and −54 (Fig. 2). When this region was searched for the presence of transcriptional signals known to occur in other eukaryotic genes, a striking homology to the TATA proximal sequences of the 70 kDa *Drosophila melanogaster* heat shock protein (*hsp70*) promoter was apparent (Fig. 4). At position −57, the sequence C--GAA---TTC--G, which differs from the consensus heat shock regulatory element only by the insertion of one extra nucleotide in the middle of the motif (Pelham 1985) is found. In addition, another imperfect homology to the HSE (6 out of 8 nucleotides) is located at position −70 overlapping the downstream HSE-like motif (Fig. 2).

Discussion

In this manuscript, we report the isolation and characterization of a genomic clone encoding the *PR-1a* protein from tobacco. Analysis of the 5′ flanking sequence of this gene at the nucleotide level revealed the presence of characteristic

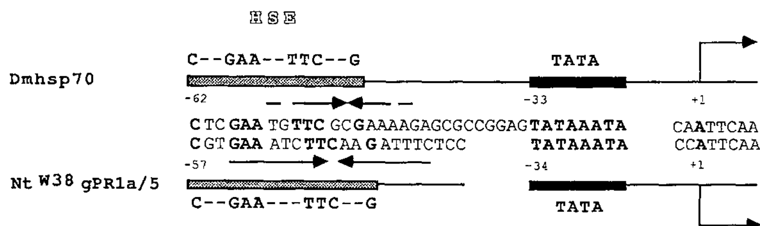

Fig. 4. Comparison of nucleotide sequences in the TATA proximal regions of the *Drosophila melanogaster hsp70* promoter (Karch et al. 1981) and the *PR-1a* gene. Gaps were introduced in the *PR-1a* sequence in order to maximize homology. Sequences matching the consensus for TATA (*black box*) and the heat shock regulatory element (*stippled box*) are shown in *bold letters*. *Horizontal arrows* mark inverted repeats. Numbering is relative to the respective transcription start sites (in *bold face*) designated +1 and the direction of transcription is indicated

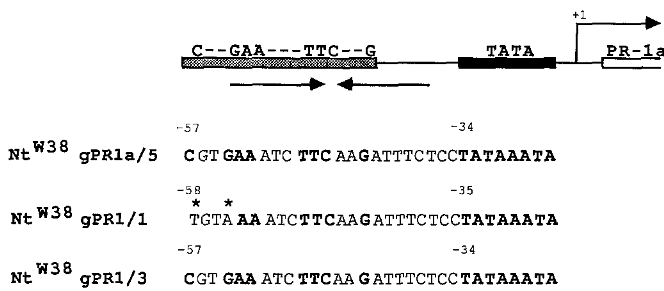

Fig. 5. Comparison of nucleotide sequences in the 5′ flanking regions of the *PR-1a* gene and two *PR-1* pseudogenes. Sequences matching the TATA consensus (*black box*) and the heat shock element-like motif (*stippled box*) are shown in *bold letters*. *Horizontal arrows* mark inverted repeats. *Asterisks* denote mismatches to the sequence of the *PR-1a* gene. Numbering is relative to the transcription start site designated +1 and the direction of transcription is indicated

eukaryotic transcription signals like TATA as well as putative regulatory sequences. Of special interest is a region of dyad symmetry (−54 to −38) located 3 bp upstream of the TATA box (−34 to −27), which contains part of a perfect match to the consensus HSE (−57 to −43), the only difference being the insertion of one additional nucleotide (Fig. 4). Identical or very similar structural elements were found at the same positions in the two *PR-1* pseudogenes analyzed (Fig. 5).

The heat shock regulatory element (HSE) is a common motif in the flanking regions of all heat inducible genes and has been demonstrated to have a positive effect on transcription (for review see Pelham 1985). A synthetic oligodeoxynucleotide containing the consensus sequence is sufficient for the activation of a foreign gene under heat shock (HS) conditions. This gene regulation is mediated by a specific protein, the heat shock transcription factor (HSTF), which binds to the HSE. In analogy, the TATA proximal motif of the *PR-1a* gene might be involved in transcriptional regulation of this gene.

The presence of a HSE-like sequence in the upstream region of the *PR-1a* gene is quite unexpected. *PR-1* proteins are known to be strongly induced by multiple environmental stimuli (for review see van Loon 1985). In this respect they closely resemble other stress proteins. Furthermore, the tobacco *PR-1* proteins as well as the plant low molecular weight HS-proteins (most thoroughly studied in soybean; for review see Kimpel and Key 1985) constitute families of highly related polypeptides. Their molecular weights are around 15 kDa and 17 kDa, respectively; once induced, the proteins are extremely stable; the respective genes are not interrupted by intervening sequences. Thus, *PR-1* proteins and HS-proteins share many features in common. On the other hand, however, there is no homology at the amino acid level between these two groups of proteins. The plant HS-proteins associate during HS with nuclei or mitochondria or they are specifically transported into the chloroplasts, whereas *PR-1* proteins are localized in the intercellular space (Wagih and Coutts 1982; Parent and Asselin 1984; Carr et al. 1987). Moreover, according to reports from other groups (Ohashi and Matsuoka 1985) and our own results, *PR-1* genes are not induced to detectable levels under HS conditions. On the contrary, it has been demonstrated that PR-protein synthesis is completely suppressed after shifting tobacco plants from 20° C to 30° C (van Loon 1975).

An observation with relevance to our finding has recently been made. A rat heat shock cognate (*hsc73*) gene, which is expressed constitutively at a high level in growing cells and is not heat inducible in vivo, contains two matches to the consensus HSE in the 5′ flanking region (Sorger and Pelham 1987). When a fusion of the *hsc73* promoter with the sequences encoding chloramphenicol acetyltransferase was assayed in HeLa cells, only a slight increase (<2 fold) in enzyme activity could be observed after HS. Deletion analysis of this construct, however, revealed that truncation of the promoter at −84 clearly resulted in heat inducibility, proving that the TATA proximal HSE can function.

The transcriptional regulation of a gene may thus be complex and mediated by several structural elements working in concert. Therefore, it might be possible that pathogen and HS responses are two distinct stress reactions in plants, related to each other by a common evolutionary origin of some elements involved in the regulatory mechanisms. Several possibilities are conceivable: (1) binding of the HSTF to the HSE-like motif might be necessary but not sufficient for *PR-1* gene transcription, (2) the insertion of one nucleotide in the middle of the HSE might eliminate binding of the HSTF to this sequence. Instead, a related protein, which specifically interacts with the HSE-like motif might be induced after pathogen invasion and activate *PR-1* gene transcription, (3) under HS conditions, HSTF might bind to the HSE-like motif and repress *PR-1* gene transcription due to steric interference with the TATA binding protein.

Analysis of further pathogen induced genes from different plant species will show if a HSE-like motif is generally associated with this response. In addition, gene transfer experiments with the functional *PR-1a* promoter fused to a reporter gene and deletion mutants of this construct should help us to elucidate whether the HSE-like motif can operate as a regulatory element, and whether upstream sequences of the *PR-1a* gene could possibly act as modulators of a HS response. Irrespective of these functional studies, our finding describes a molecular link between heat shock and pathogen stresses in plants.

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