Two *PR-1* Genes from Tomato Are Differentially Regulated and Reveal a Novel Mode of Expression for a Pathogenesis-Related Gene During the Hypersensitive Response and Development

Pablo Tornero, José Gadea, Vicente Conejero, and Pablo Vera

Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universidad Politécnica-Consejo Superior de Investigaciones Científicas, Camino de Vera s/n, 46022-Valencia, Spain

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Pathogenesis-related (PR) proteins form a heterogeneous family of plant proteins that are likely to be involved in defense and are inducible by pathogen attacks. One group of PRs, represented by the subfamily PR-1, are low-molecular-weight proteins of unknown biochemical function. Here we describe the cloning and characterization of two closely related genes encoding a basic and an acidic PR-1 protein (*PR1b1* and *PR1a2*) from tomato (*Lycopersicon esculentum*). We present a comparative study of the mode of transcriptional regulation of these two genes in transgenic tobacco plants using a series of promoter-GUS fusions. Unexpectedly, the chimeric *PR1a2/GUS* gene is not induced by pathogenic signals but instead shows constitutive expression with a reproducible developmental expression pattern. It is expressed in shoot meristems, trichomes, and cortical cells as well as in vascular and nearby tissues of the mature stem. This constitutive expression pattern may represent preemption of plant defenses against potential pathogens. Conversely, the chimeric *PR1b1/GUS* gene does not show any constitutive expression in the plant, but it is transcriptionally activated following pathogen attack. Upon infection by tobacco mosaic virus, the *PR1b1* gene is strongly activated locally in tissues undergoing the hypersensitive response but not systemically in uninoculated tissues. Furthermore, its expression is induced by both salicylic acid and ethylene precursors, two signals that coexist and apparently mediate the activation of local defenses during the hypersensitive response. We speculate that the different mode of expression of the two genes presented here, together with that reported previously for the induction of other *PR-1* genes in systemic, uninoculated tissues, may all be complementary and necessary for the plant to acquire an efficient refractory state to resist pathogen attacks.

Additional keywords: SAR, systemic acquired resistance.

Plants have evolved a bewildering array of synergistic mechanisms to defend themselves against pathogenic attacks. Some of these mechanisms are established in the plant before the arrival of the microbe, whereas others are inducible upon the perception of pathogenic signals and can protect the plant not only at the site of infection but also systemically, throughout the plant. All these mechanisms constitute the basis for the natural immunity of plants to many pathogens.

One form of this inducible resistance results in the restriction of pathogen growth to tissues immediately adjacent to the infection site and is mediated by the triggering of local cell death. This localized cell death, manifested in most cases as a visible necrotic lesion, is called a hypersensitive response (HR) (Matthews 1991). The onset of an HR normally precedes the activation of a signaling process throughout the plant that makes it more refractory or resistant to subsequent infections by a broad spectrum of pathogens. This response, called systemic acquired resistance (SAR) (Ross 1961), is common in many incompatible plant-pathogen interactions. During this response, the plant selectively and coordinately activates the expression of a plethora of genes (SAR genes) encoding proteins related to defense (Ward et al. 1991). A subset of these proteins is known as pathogenesis-related (PR) proteins and comprises several gene families, consisting of basic and acidic subfamilies, which appear to locate in the vacuole or extracellularly (Cutt and Klessig 1992). Expression of genes encoding PR proteins has also been found to be induced in dying tissues which are in direct contact with the pathogen and are thus developing HR. Functionally, some PR proteins have been characterized as having antifungal activities in vitro or in vivo, such as chitinases (i.e., PR-3) or β -1,3-glucanases (i.e., PR-2) (Broglie et al. 1991; Mauch et al. 1988; Zhu et al. 1994), while the biochemical and biological function of other PRs has yet to be identified. The demonstrated antimicrobial activity of some PRs and the fact that they are induced during the HR and SAR responses lead to the suggestion that PRs are at least partially responsible for maintaining the disease-resistant state (Lawton et al. 1993).

A particularly interesting PR gene subfamily is that encoding PR-1 proteins which have an unknown biochemical function. In tobacco, both basic and acidic PR-1 proteins have been identified on the basis of serological relations and on se-

Corresponding author: P. Vera; Phone: 34-6-3877864; Fax: 34-6-387-7859; E-mail: vera@ibmcp.upv.es

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quence analyses. Transgenic plants constitutively expressing the acidic PR-1a isoform gene have increased tolerance to the fungal pathogens *Phytophthora parasitica* and *Peronospora tabacina* (Alexander et al. 1993), implying that this gene family, or at least some members of it, can play a role in disease resistance. In tobacco, expression of basic and acidic isoforms of PR-1 is differentially regulated. *PRB-1*, a basic-type PR-1 gene, is activated by the plant hormone ethylene and shows constitutive expression in phloem tissues of the petiole and pedicel abscission zones (Eyal et al. 1993). Conversely, *PR-1* genes encoding acidic counterparts from tobacco are induced during the HR and at the onset of SAR and are induced by salicylic acid (SA), a possible endogenous signal molecule mediating the SAR response (Ohshima et al. 1990; Van der Rhee et al. 1990; Uknes et al. 1993; Yalpani et al. 1991).

However, in spite of the well-characterized *PR-1* gene family in tobacco, scant information is available on whether and how this mode of gene regulation is generally established for homologous genes from other plant species. In *Arabidopsis*, two *PR-1* genes have been described; one of them is not induced by pathogen attack (Metzler et al. 1991) and the other is generally used as a molecular marker of SAR (Uknes et al. 1992). In tomato plants, at least three cDNA clones encoding different PR-1 isoforms have been described and shown to be induced under different pathogenic situations (Tornero et al. 1993, 1994; Van Kan et al. 1992), but no data exist on the ge-

nomical organization and the transcriptional regulation governing the expression of different members of this gene subfamily from tomato. Towards this end, we report here on the isolation of genomic clones of the *PR1b1* and *PR1a2* genes from tomato, which encode a basic and an acidic PR-1 isoform, respectively. We introduced different promoter-GUS fusions of these two different *PR-1* genes into tobacco and performed a comparative study of their mode of expression during development and in response to infection by tobacco mosaic virus (TMV) and chemical inducers of defenses. Interestingly, the newly described *PR1a2* gene is developmentally regulated and shows no induction over basal constitutive levels upon TMV infection. Conversely, the *PR1b1* gene is induced by pathogenic signals and shows neither constitutive expression nor developmental regulation.

RESULTS

Cloning and characterization of genes encoding an acidic and a basic PR-1 protein from tomato.

The cDNA encoding a previously described basic PR-1 protein in tomato (Tornero et al. 1993) was used as a hybridization probe to screen a genomic library from tomato plants. As a result of this screening, two different lambda clones (λ -17.3 and λ -R3) were isolated and characterized by restriction endonuclease site mapping and DNA sequencing. A 2.4-kb DNA

PR1b1

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CATATCAAACTATTTATGCTATATTTAATCACTTATTTAAATCGTAACATATTTATTTCTAA -881
ATTGCGAGCTCTAACACTTTTAAAAAGTTTAAAAATTTCTTTTGAAGCTCAACAGGATT
GAGGCGATTTCTCAATTTCCACATTAaaggaccacaaatTTGTTTTCGTTAAAGTGTGCTA -761
TCACCTCCATTTGTTGTTGTTTTCATTTATATCCATTTATATCCAAATTCCTTCAATGACT
AACAAATACCTTGAGTCTTCTCTCTCTCATAGATAAATCTTCTCCATAAATCCACGTAAG -641
gagagcCAATTAAGTGTGTTTAAATATTTCTCAAAATTAAGATAAGTATGATTATTTCTT
TAAAGCATATAGTATTTCTTAAATCAACAGCATGCAATCTCTTTTGAATTTTCTTTCACA -521
TGTAATATAAATTCACAAAGTTTAAATTTAAACAAATTTTCACAAATTTGATTAATTTGAA
CAATCAAAAGTTTGATAAATATCTTATCGAAACCAAAAGTGTTCACATTTTGAAAAATATAa -401
aggaccacaaatAAGATCACTTTTGACAAATCTTAAggaccacaaatTTGCTCTTATCATACATA
AGGGAGTCTAATGAACTTACATAAAAGATAAGGAGGCTTTTGTCTATTTCTCTATAATTT -281
TCCCTTTGAATTTTCTTCAATACATGCTTAAACAAATTTACACAATGTTTAAATATATATAT
ACTATTTAAaggaccacaaatGTAATTTAATTTCTAAAGTTTAAggaccacaaatTCACCTTAG -161
CAAAATACACAAGAGACTATTTTGAAGCTTACTATCAAGATAAGAGACCATTTTATCAT
TTCTCTACTAATAATTTCTCTTGAATTTTCTTTCACACATCTTAGCAAAATTCACCAAT -41
TTTTACTTataaagTACACTATTTCTACGTTTATAATCTACAAATACCTTAGATTTATTT 20
TCTCTCACTAAATCAAGAAATAGGGGTTGTTCAACATCTCATTTGTACTCTGTT 80
M G L F N I S L L L T C 12
CTCATGGTATTAGCCATATTTCACTCTTTGAGGCCCAAAATTCACCCCAAGTACTCT 140
L M V L A I F H S C E A Q N S P Q D Y L 32
CGCGTTCAACAGATGCCCTGCCAAGCTCGAGCTCGGCGCTATGCTTGGGATGCCAAC 200
A V H N D A R A Q V G V G P M S W D A N 52
TTGGCATCCGAGCACAAATATGCCAATCAAGAGCTGGTGATTGTAACCTTGATTCAT 260
L A S R A Q N Y A N S R A G D C N L I H 72
TCTGGTGTCTGGGAGAACTCTGCCAAGGGTGGTGGTCTACGCGGAGGGCAGCGCTG 320
S G A E N L A K G G G D F T G R A A V 920
CAATTTGGTGTCTCGAGAGGCCAAGCTATACTACGCTACCAACCAATGTCTTGGTGG 380
Q L W V S E R P S Y N Y A T N Q C V G 112
AAAAAGTTAGACATTTATCTCAAGTAGTCTGGGCAACTCAGTCCGACTAGGTTGTGGT 440
K K C R H Y T Q V V W R N S V R L G C G 132
CGGGCAGCTTGCAACAACGGATGGTGGTTCATTTCTTGCAACTATGATCCTGTAGGCAAC 500
R A R C N N G W W F I S C N Y D P V G N 152
TGGATCGGACAACGCTCTTACTAAAATGATGTATCTTATGACATGTTGCTAGTATTA 560
W I G Q R P Y * 159
TAAATTTCTCATATGAGACGTCGAGCACTTAAATTTAAAGTTTGCACATATGAATCAAGTC 620
AAACTCTATCTAAAAATATTAAGGATTAATATTTGAACATCTATAATTTATTTATTTCT 680
CCTTTTGTATGTTGCTATATGATAATTAATTTCCACATACCATATGTTTCATATTTGGGCTTAA 740
TGATATTAAGTACTGCACTCTCTGTTTTCATTAACAAATTAATATACATAAAATTTTAA 800
TTAAGCATTCAGATATATTAATTTAGGCACATCAAGCACTTATCTAAACAGCTAATATTTAA 860
TTCAATAATTCGATTTCAACCAATTTGTTCTATTTTAAAGTGGCCATCAAAA 920
AAAATTCACATTTACCAATTTTCTTCAATGACTACCAAAATCTCAAGTCTTCCATAT 980
TCTCTCATACAAATTTGGCCATAAACTATATTTTCATAAAATATTTTATAGGTGAAAAT 1040
GAATTTTCACTCTTCTTGACCTATTTGCTTTAGATTTGAATACCAAGAAATAAACCAAT 1100
TTGCTATTTATTTGAAAAATTTTACGTAGCATGAAATTTAAATTAATTTGGCACATTTTAAA 1160
ATATCTCTGTCAGGAAAAAAATTTGTAACCCCTTAACAAAGCTTTAAATTTAATTAATTTTCA 1220

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PR1a2

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AGTTGGACATAGATCTCTTATTTCTTTAGAAATATAACGTATTCACAAATTTTAAAG -900
TGGTCTGTAATTAATAACAAAGTCAAGAAACTCTTCTTCAAAATGGAAATGGAATTCAC -840
CGCATCAGTCTTTGAACACTTTAACTTTTAAATTTGAAAAATATGATACATATATCCGTT -780
CAAAACACTCTTTATGCGCATATATATTTGATTAACCATATCAATTTTCTTATCTCTAC -720
TTATTACCTTATGTAAACACCTTAACTAATTTATTCATAAATTCACATTTCACTCTCTTAA -660
ACGTTTAAAAATTAATTAATTTCCCAATTTGAAGCAGCCATGATGAGGCGAGTTAATTTCT -600
ACACAAAGAACCCATATTAAGTGTCCACACTTTGTTTATATGTTTTCATTTATATCCAA -540
ATTGCTTAAATTTCAATGACTAACAAAGAAATTTTCTTTTCATCATTTCAAGGCGGCT -480
CAATAATATTGATGACCCCAAAATCAATTTGTCATAAAATATTTTATAGTAAAAACTGAA -420
TTGCTATCTCTTTTCTTATAGAGGTTTGAATACAAACCCGAAACATAGATAAATATTAT -360
TTAGATTGAAATTTTCTTTAAAAAGCTTACGGTGATGCAAAATTAATTAATTAAGACTC -300
CAAAACACATTTGCAATATCTAAATTAAGATGACAAATTTTAAAGTATATATAGTAA -240
TCCCTAAATCAACAGATATGGAAGGTATGATCAGATTTGAAATTTCTTCACATATTTAA -180
TTCACATGTTTGAATTAATCAATCAAAAGTTAGATATAAGAAAAAAATATTTATTTT -120
TGAAAAAATTTAAAGGACCAAAATGTAATTTAATTTTAAAGGTTTAAggaccacaaatTIG -60
CTTAGCAAAATTTCCCAATTTTACTTataaagTACACTACTTATCTACATTTTATAATCTA 1
CAACACTTAAATTTTATTTTCTCTCTCAAGCAAAATAGGGGTTGTTTAACTATGCTATGTT 61
M G L F N M S L L R 121
ACTTATGACTTGTCTCATGTTATAGCCATATTTCACTCTTGTGATGCTCAAAATTCACC 129
L M T C L M V L A I F H S C D A Q N S P 29
CCAAGACTCTTGGGTTTCAACACGCGCGTGGGCTGCGAGTGGGCGCAATCTC 181
Q D Y L E V H N D A R A Q V G V G P M S 49
TTGGGATGCCGACTTGAATCCCGAGCACAAAGCTATGCCAATCAAGAGCGGGTGATTTG 241
W D D A L E S R A Q S Y A N S R A G D C 69
TAACTTGATTCATTTCTGTTTCAAGGAGAACTCTGCCAAGGGTGGTGGTACTTTCACGGG 301
N L I H S G S G E N L A K G G G D F T G 89
GAGGCGCGCTTGAATTTGGGTTGTCGGAAGGCAAACTACCAATCAGATCAGATGA 369
R A V E L W V S E K P N Y N D T N E 101
ATGTTTAGCGAAAAATGTGCGGACATTATATCTCAAGTAGTCTGCGGTGACTCAGTTCC 421
C V S G K M C G H Y T Q V V W R D S V R 129
ACTAGGTTTGGTGGGCTCTTTGCAACGACGGGTGTTTATTTCTTGCAACTATGATCC 481
L G C G R A L C N D G W F I S C N Y D P 149
TGTAGGCAATTTGGTTCGGAACGCTTACTAAATGTTTCTTTTCTTATGATGTTGTA 541
V G N W V G Q R L T K M F F F L Y D V * 168
AGGATCAAAATTAATTTATTAATTTCTTTGATCTTTGCTACTATGAATTAATTCACATTA 601
CCATATTTGTCATGTTATAGTGGGCTTAAAGTTGATAAATAAAGGTTTCTTCTATTTTATTT 661
AAATAAGATTAATAATACATGGCATATGTTGTAATAATTTATTTATTTGCTTATGCAAAAT 721
CGAGAGTTAAATTTATTAATTTTAAAGTGAAGACGATCAATGATTTCTTATATCTTAA 781
TAAATGAATTTATATTTTAAAGCGGTTTGAATGATATATAAGTAGAGCGGATCTAG 841
GATTTCAAGGTAAATGGGTTGATATCGTTAAGTCTCAATTTGTTGTTTCTTATGATTTTCA 901
TATGCAATTTATATTAATGTTTATTAAGTCAACAGTATGCTTATGATTTTATGTTATTA 961
ACATTTGCTTGTATGTTTGGATTTAGCGTTTGAAGTGAAGCGTCAACATTTATTTTGTCA 1021
AAAGAAATGGAAGGATCAATTAATTAAGCAATTAAGAAATGAACGACGATGCTGCTCA 1081
CTTGTGTCAGGGGTGACAGATTTAGTTTTCATTAAGTTTATATGATCAAAATATATACAT 1141
ACACATGTAATACATAAAATTTCAACCAAGATCAITGGATGCGGTGACACCTACCATGAT 1242

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Fig. 1. Nucleotide and deduced amino acid sequences of the *PR1b1* and *PR1a2* genes. The deduced amino acid sequence is given below the nucleotide sequence. The transcriptional start sites are indicated in bold. Putative TATA boxes are in underlined lowercase. The GGCGGCTC motifs are in underlined lowercase italics. The conserved and repeated sequences motifs (AAGGACCAAAA) in the promoter regions of these two genes are in lowercase.

fragment from λ -17.3, identified by hybridization with the cDNA, carried the complete coding region. Part of the assembled nucleotide sequence of this DNA fragment is shown in Figure 1. This DNA segment contains a single open reading frame (ORF) preceded by 985 bp of 5'-flanking DNA and followed by 1,400 bp of 3'-flanking DNA. A computer-based DNA comparison indicates that this *PR-1* gene encodes the bona fide pathogen-induced mRNA represented by the cDNA clone pTE28.1, which encoded the basic PR1b1 isoform (Tornero et al. 1993). The transcriptional start site of the *PR1b1* gene was determined by primer extension using a 5'-end-labeled primer complementary to nucleotides +76 to +97 and mRNAs from tomato plants treated with ethephon (data not shown). Through this analysis, the predominant 5' terminus (position +1) was localized to the A residue 44 nucleotides upstream of the initiator ATG codon (Fig. 1). A putative TATA box is found at position -33, and a CAAT-like sequence resides at -44. At the 3' end of the gene, different consensus sequences for polyadenylation are found.

Similarly, a 3-kb DNA fragment from λ -R3 carried the complete coding region for a novel PR-1 protein member, which was designated PR1a2 because of the acidic pI of the deduced polypeptide (see below). Part of the assembled nucleotide sequence of this DNA fragment is shown in Figure 1. The DNA segment contains a single ORF encoding the PR1a2 polypeptide. The ORF is preceded by 1,600 bp of 5'-flanking DNA and is followed by 1,400 bp of 3'-flanking DNA. By analogy to the *PR1b1* gene described above, the transcriptional start site of the *PR1a2* gene was assigned to the A residue 35 nucleotides upstream of the initiator ATG codon (Fig. 1). A putative TATA box is similarly found at position -33, and a CAAT-like sequence resides at -44. At the 3' end of the gene, two different consensus sequences for polyadenylation are found.

The encoded PR1a2 polypeptide has 168 amino acid residues and contains an N-terminal signal peptide of 25 amino acids that after cleavage would result in a mature protein of 143 amino acids with an estimated molecular weight of 15,886 and an acidic pI of 4.92. Comparison of the derived amino acid sequence of this new acidic PR1a2 with PR1b1 and with different PR-1 proteins previously identified in tomato and other plant species revealed high identity in the coding regions (data not shown). Interestingly, the newly identified PR1a2 was more homologous to the basic PR1b1 (87% identical amino acid residues) than to the previously identified acidic PR1a1 isoform (51% identical) from tomato (Tornero et al. 1994). The amino acid sequences of the deduced PR1b1 and PR1a2 proteins are compared in Figure 2A. Most of the amino acid changes observed correspond to single nucleotide substitutions in the coding regions, indicating that the homology between these two proteins is even higher at the nucleotide level (92% identical). PR1a2 contains a carboxy-terminal extension, relative to the basic PR1b1, which presumably could dictate the final localization of the protein, as has been suggested for other PR proteins (Neuhaus et al. 1991).

Computer-aided nucleotide comparison indicated that the high identity existing in the ORF regions can be extended to the proximal 5' promoter regions of the *PR1b1* and *PR1a2* genes (50% identity over a stretch of 850 nucleotides). Sequences were identified which are highly conserved if not identical in these two promoters. This homology is highest in the promoter regions close to the initiator ATGs and extends

along a stretch of 150 nucleotides upstream of the ATG initiation codons (Fig. 2B). A conserved region of 44 nucleotides containing a direct repeat of the 11-nucleotide sequence AAG-GACCAAAA is separated by 21 and 22 nucleotides in the *PR1b1* and *PR1a2* promoters, respectively (Fig. 2A). This 11-bp sequence is found three more times along the promoter region of the *PR1b1* gene (Fig. 1), at positions -368, -400, and -795, with similar spacing between the first two of these (Fig. 1). Additionally, the 8-bp conserved sequence motif GCC-GCCTC is found at positions -641 to -634 of *PR1b1* and at positions -487 to -480 of *PR1a2* (Fig. 1). Interestingly, promoters of ethylene-inducible PR genes from diverse dicotyledonous species contain this 8-bp consensus sequence. In *Nicotiana*, this core sequence is present as a direct or inverted repeat and is necessary for induction of the corresponding PR genes by pathogenic attack or by ethylene (Meller et al. 1993; Alonso et al. 1995; Eyal et al. 1993 and references therein). Besides this element, no other conserved motifs were found in any of the two promoters under consideration.

The lack of more significant homology in the promoters of these genes, encoding for closely related proteins, is in agreement with their completely different mode of transcriptional regulation (see below).

Developmental and tissue-specific regulation of the *PR1a2* and *PR1b1* gene promoters.

Chimeric *PR1b1/GUS* and *PR1a2/GUS* reporter constructs were introduced into tobacco and used to study gene expression. The chimeric *PR1b1/GUS* gene was constructed by fusion of the region from nucleotide -914 to nucleotide +56 of

A

PR1a2	MGLFNMSLLMLTCLMVLAIFHSCDAQNSPDYLEVHNDARAQVGVGPM	50
PR1b1	-----I-----E-----A-----	49
PR1a2	DAQLESRAQSYANSRAGDCNLIHSGSGENLAKGGDFTGRAAVELWVSEK	150
PR1b1	--N-A--N-----A-----Q-----R	149
PR1a2	PNYNYRTNECVSGMKMGHYTQVWRDSVRLGCGRALCNDG.WFISCNYP	199
PR1b1	-S--A--Q--G--R-----N-----R--N-W-----	199
PR1a2	VGNWVGQRLTKMFFFLYDV	168
PR1b1	----I---PY	159

B

PR1b1	-339	AATGTTTAAATATATATACACTATTTAaaggacacaaaGTAATTAATCT	-289
PR1a2	-132	AATATTCATTTTGAAGAACTTAAaaggacacaaaGTAATTAATCT	-83
PR1b1	-285	AAAAAGTTTAAaggacacaaaTCACCTAGCAAAATACACAAGAGACTATT	-139
PR1a2	-82	AAAAAG.TTTAaggacacaaaT.....	-62
PR1b1	-138	TTGAACCTACTATCAAGATAAGAGACCATTTTTATCATTTCTCTACTA	-90
PR1a2			
PR1b1	-89	ATAATTCCTTTGAATTTCTTCACACACTACTAGCAAAATTCACAATT	-40
PR1a2	-61	TGCTTAGCAAAATTCACAATT	-40
PR1b1	-39	TTTACTTATAAATACACTACTTATCTCAGCTTTATATATCAATAACTTA	+11
PR1a2	-39	TTTACTTATAAATACACTACTTATCTCAGCTTTATATATCAACAACCTTA	+11
PR1b1	+12	GATTATTTTCTCTGCACTAAACCTAAGAAAAATG	+47
PR1a2	+12	AATTTATTTTCTCT.....CAAGCAAAAATG	+38

Fig. 2. A, Comparison of the amino acid sequences of the deduced PR1a2 and PR1b1 proteins. **B**, Comparison of the nucleotide sequences of the 5' proximal promoter regions in the *PR1b1* and *PR1a2* genes.

PR1b1 to the ATG initiation codon of the GUS coding sequence and the 3' nopaline synthase (*NOS*) regulatory sequences (Fig. 3) of the binary vector pBI101 (Jefferson et al. 1987). The chimeric *PR1a2/GUS* gene was constructed similarly by fusion of the region from nucleotide -1,600 to nucleotide +47 of *PR1a2* to the ATG initiation codon of the GUS coding sequence in pBI101. Site-directed promoter deletions at nucleotide -418 of *PR1b1* and nucleotide -334 of *PR1a2* were also fused to GUS (Fig. 3) and introduced into *N. tabacum* cv. Xanthi.

To study the spatial distribution of GUS activity driven by these *PR1* promoters during different developmental stages of growth, GUS activity was determined in seedlings and in full-grown plants. GUS activity as revealed by fluorometric determination was five to 30 times higher in stems, roots, and leaves of individual plants harboring the *PR1a2/GUS* construct than in control (untransformed) plants, but the transgene retained the same spatial and temporal pattern of expression in all the independent transformants, as revealed by histochemical detection of GUS. The resulting pattern of expression observed in different transgenic lines is summarized in Figure 4. *PR1a2/GUS* expression was initially detected in shoot meristem or in meristematic regions of axillary buds soon after true leaves appeared on the plant (Fig. 4C). This meristem-specific expression was maintained in full-grown plants (Fig. 4D). As the seedlings developed and enlarged, the transgene was observed to be expressed gradually along the vascular strands of petioles and leaves (compare Fig. 4A and B). This vascular-specific expression was also observed in fully expanded leaves, where GUS activity was specifically detected in major and minor veins all along the leaf blade, as well as in trichomes (Fig. 4H and I). In petioles the expression was associated with trichomes and subepidermal cortical cells, which became intensively stained for GUS activity (Fig. 4G and H). In the central axis of a mature plant, GUS activity was localized, prominently, in the node regions. Here (Fig. 4E and F), it appeared as if the *PR1a2/GUS* transgene followed a gradient mode of expression along vascular strands emanating from the node. The expression was highest in the central vasculature where the leaf trace connects, as well as in the external (adaxial) cortical cells and trichomes. Along the internodes of the stem of a full-grown plant, *PR1a2* promoter expression was gradually activated as the stem matured. Here, GUS activity

was associated externally with cells of the cortex and internally with pith parenchyma cells in internodes that had initiated secondary growth as well as in ray parenchyma cells of the differentiated xylem tissue (Fig. 4J and K). In the younger internodes, which were closer to the shoot apex and apparently had not yet initiated secondary growth, no GUS activity could be detected, in contrast to the highly localized expression in the shoot meristem (Fig. 4D). In roots, GUS activity was detected in the older parts (e.g., where the main root merges with the stem), while in flowers GUS activity was nearly undetectable, if not absent (data not shown).

This developmental pattern of expression was maintained, albeit with some reduction in the intensity of GUS activity (particularly in trichomes), in transgenic plants harboring the (-334)*PR1a2/GUS* deleted construct (not shown).

In marked contrast, similar analysis of transgenic plants harboring the (-914)*PR1b1/GUS* construct or the deleted (-418)*PR1b1/GUS* version revealed no constitutive expression in any of the tissues analyzed above (data not shown) but rather showed a reproducible and predictable induction upon pathogen attack or SA treatments, as expected for a bona fide PR gene (see below).

TMV-induced expression of the chimeric *PR1b1/GUS* but not the *PR1a2/GUS* in transgenic tobacco plants

TMV-mediated induction has been confirmed in transgenic tobacco plants harboring promoter-GUS fusions of endogenous acidic PR-1 genes (Van der Rhee et al. 1990; Oshima et al. 1990). Because of the parallelism in the existence of *PR-1* genes in tobacco and tomato, we comparatively studied the effect of TMV infection on the expression of *PR1b1/GUS* and *PR1a2/GUS* in transgenic tobacco plants. A summary of the assay data in some of these transgenic lines is given in Table 1. Transgenic plants containing (-914)*PR1b1/GUS* showed a 20- to 100-fold induction of GUS activity in leaves at 60 h after infection with TMV and showing the characteristic HR. No detectable induction was observed in the upper, uninoculated leaves of the same plants 6 to 8 days after inoculation with TMV. Transformants containing (-418)*PR1b1/GUS* had apparently lost the intense inducibility by TMV infection and only underwent a twofold to 12-fold induction in inoculated leaves. Negligible GUS activity was found in mock-inoculated plants or control plants lacking the chimeric GUS constructs.

Fig. 3. Chimeric *PR1b1/GUS* and *PR1a2/GUS* genes used in this study. The acceptor plasmid for all the promoter fragments tested was pBI101.1. Numbers indicate the distance (in base pairs) from the proximal transcription start site (+1). The relative positions of the putative regulatory element (GCCGCC) discussed in the text are shown in shaded boxes. The TATA boxes are indicated by an empty rectangle.

These GUS activity data allowed us to conclude that some sequences between position –914 and position –418 upstream of the *PR1b1* coding region are necessary for maximal induction during the HR induced by TMV.

Similar experiments performed with transgenic tobacco plants harboring either (–1,600)*PR1a2*/*GUS* or (–334)*PR1a2*/*GUS* revealed an opposite pattern of expression for the chimeric gene (Table 1). Upon TMV infection, no induced expression over basal levels of GUS activity driven by the –1,600 *PR1a2* promoter could be found in the TMV-inoculated leaves which were undergoing HR, but instead a slight reduction with respect to mock-inoculated plants was observed in tissues showing necrotic lesions. In the upper, uninoculated leaves, which were to develop the SAR response, no apparent variation in the expression pattern was observed with respect to mock-inoculated plants. Similar results were obtained when transgenic plants containing the deleted (–334)*PR1a2*/*GUS* gene were inoculated with TMV (Table 1).

This different mode of expression was further confirmed by direct visualization of GUS activity in intact tissues. In all the

kanamycin-resistant progenies analyzed that harbored the chimeric (–914)*PR1b1*/*GUS* construct, GUS activity was activated during the HR to TMV infection. The gene was expressed at high levels in the tissue immediately surrounding a

Table 1. Local and systemic induction of GUS activity in transgenic tobacco plants harboring promoter-GUS fusions after TMV infection^a

Construct	Mock-inoculated plants	TMV-infected plants	
		Inoculated leaves	Uninoculated leaves
(–914) <i>PR1b1</i> / <i>GUS</i>	200	16,400	350
(–418) <i>PR1b1</i> / <i>GUS</i>	90	2,060	130
(–1,600) <i>PR1a2</i> / <i>GUS</i>	3,000	2,600	3,200
(–334) <i>PR1a2</i> / <i>GUS</i>	2,200	1,800	2,000

^a GUS activity was analyzed fluorometrically as described in Materials and Methods; the data are expressed as pmol of 4-methyl umbelliferone/min per mg of protein. GUS activity was determined in F₁ progeny of three independent transformants for each construct at 72 h after mock inoculation or inoculation with TMV. The data represent the average GUS activity observed in two plants of each transgenic line.

Fig. 4. Localization of GUS activity in transgenic tobacco seedlings transformed with the (–1,600)*PR1a2*/*GUS* construct. **A–C**, Seedling stage. **D**, Longitudinal section of shoot apical meristem of a full-grown plant. **E**, Longitudinal section of a mature node. **F**, Cross sections of a node like that in **E** at two successive stages of leaf trace connection. **G** and **H**, Petiole cross section. **I**, Magnification of the adaxial leaf surface. **J** and **K**, Internode cross sections at two different stages of stem maturation: intermediate internode (**J**) and internode at the base of the plant (**K**). am = Apical meristem; c = cortex; e = epidermis; iph = internal phloem; lb = lateral bud; oph = outer phloem; p = pith parenchyma; t = trichomes; v = vascular tissue; x = xylem.

local lesion, as indicated by distinct blue staining around each lesion at 60 to 72 h after inoculation (Fig. 5A, left). Later (4 to 5 days after inoculation), when necrotic lesions were fully developed, GUS activity was detected at greater distances from

the lesion and in a more diffuse manner (not shown). Similar analysis in transgenic tobacco plants expressing the deleted $(-418)PR1b1/GUS$ showed that the transgene was still activated following TMV inoculation, but the extent of induction

Fig. 5. Localization of TMV-induced and chemically induced expression of the GUS gene in transformed plants. **A**, GUS expression in leaf tissue 3 days after inoculation with TMV: plants containing $(-914)PR1b1/GUS$ (left) and $(-418)PR1b1/GUS$. **B**, GUS expression in leaf tissue 3 days after inoculation with TMV: plants containing $(-1,600)PR1a2/GUS$ (left) and $(-334)PR1a2/GUS$. **C–F**, Leaf disks from transformants containing $(-914)PR1b1/GUS$ (**C**), $(-1,600)PR1a2/GUS$ (**D**), $(-418)PR1b1/GUS$ (**E**), and $(-334)PR1a2/GUS$ (**F**) 48 h after flotation in buffer (disk 1), in buffer plus 0.5 mM salicylic acid (disk 2), in buffer plus 0.5 mM ethephon (disk 3), or in buffer plus 0.5 mM H_3PO_3 (disk 4).

was notably reduced (Fig. 5A, right). GUS activity was not found in the upper, uninoculated leaves of plants with either of these two *PR1b1* chimeric genes at least 6 to 8 days after inoculation (data not shown). TMV infection of transformants containing the chimeric (–1,600)*PR1a2/GUS* or (–334)*PR1a2/GUS* revealed no superimposed induction of GUS activity over the already established expression pattern. For these two constructs no activation was observed in the tissue immediately surrounding a local lesion at any time during the infection process (Fig. 5B). Thus, during infection and around the infection site the basic *PR1b1* gene is strongly expressed, while the acidic *PR1a2* gene is unaltered or partially down-regulated.

The *PR1b1* but not the *PR1a2* promoter activity is enhanced by SA and ethylene precursors.

SA is one of the possible endogenous signal molecules mediating the SAR response in tobacco and has been shown to be increased in tissues undergoing TMV-induced HR. Also, when applied exogenously, SA induces the expression of acidic PR genes in tobacco (Ohshima et al. 1990; Van der Rhee et al. 1990; Uknes et al. 1993; Yalpani et al. 1991; Ward et al. 1991).

Initial experiments using a leaf disk assay (Van der Rhee et al. 1990) were performed to determine the effect, if any, of SA and the optimal concentration of SA for achieving maximal induction of the chimeric genes. Leaf disks were treated with increasing concentrations of SA for 48 h at room temperature, harvested, and assayed for GUS activity. GUS activity increased linearly with increasing SA concentrations up to 4 mM for the full *PR1b1* promoter (data not shown). At higher concentrations of SA, the measured GUS activity declined, and it was almost completely inhibited by 10 mM SA. Treatment of disks with SA at concentrations below 0.02 mM produced no detectable induction of GUS activity. The expression of the deleted (–418)*PR1b1/GUS* construct was notably reduced after SA treatment, compared to that of the full promoter (Table 2). As was the case with TMV infection, these results suggest that regulatory regions governing the SA-mediated induction lie between position –1,000 and position –418. Conversely, the expression of full or deleted promoter-GUS fusions of the acidic *PR1a2* gene was not altered, compared to that of control disks treated with buffer (Table 2).

Histochemical staining of leaf tissues that had been treated with 0.5 mM SA confirmed these differences. The basic (–914)*PR1b1/GUS* transgene was mainly expressed in leaf parenchyma tissues but only slightly in leaf veins, which showed very weak blue coloration (compare disks 1 and 2 in Fig. 5C). The deleted (–418)*PR1b1/GUS* gene showed a drastic reduc-

tion in the induction level upon SA treatment (disks 1 and 2 in Fig. 5E). As expected, no effect of SA was observed in the full or deleted *PR1a2/GUS* transgenes, in which expression was restricted to vascular bundles of the leaf (disks 1 and 2 in Fig. 5D and F).

Northern blot analyses of tobacco have revealed that genes encoding PR proteins with a basic pI are expressed upon treatment with the ethylene-releasing compound ethephon (2-chloroethylphosphonic acid) (Brederode et al. 1991; Linthorst et al. 1993). Moreover, Eyal et al. (1993) have described the characterization of a basic-type *PR-1* tobacco gene (*PRB-1b*) belonging to an ethylene-responsive gene subfamily whose regulation is distinct from that of the acidic-type *PR-1* genes. The ethylene-induced expression of *PRB-1b/GUS* constructs, when assayed in transgenic tobacco plants, is mediated by the presence of an 8-bp *cis* element (GCCGCCTC) in the minimal promoter of this gene. This element is present in promoter regions of other genes encoding different basic PR proteins from tobacco (Eyal et al. 1993 and references therein) and is also found in the promoters of the two tomato *PR-1* genes presently described. This indicates that ethylene may be a signal for the specific expression of these two *PR-1* genes.

To test this possibility, we treated leaf tissue of transgenic plants with the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) or with ethephon. Ethephon (0.5 mM) or ACC (0.1 mM) activated the expression of the *PR1b1* gene (Table 2). As was the case for SA-mediated induction, ethephon induced the expression of the *PR1b1/GUS* transgene which was localized in the parenchymatic tissue and not in the veins (disk 3 in Fig. 5C). Similar observations were made after ACC treatment (not shown). Ethylene precursors had no effect in the (–418)*GUS* construct, which lacks the GCCGCCTC element (Table 2; disk 3 in Fig. 5E). Control experiments of leaf disks treated with buffer alone, 0.5 mM phosphonic acid (H_3PO_3), or hydrochloric acid (HCl), which are breakdown products generated during the conversion of 2-chloroethylphosphonic acid to ethylene (Yang 1969), resulted in no induction of the gene (Table 2; disk 4 in Fig. 5C and E). Conversely, expression of the acidic *PR1a2/GUS* constructs (Table 2) was unaffected by ACC or ethephon, and the expression remained restricted to previously established tissues (e.g., leaf veins) (Fig. 5D and F), suggesting that the 8-bp *cis* element is nonfunctional in this promoter context.

All these results suggest that both ethylene and SA, either produced by TMV infection or applied to tissues, are potential pathogenic signals which act positively on the expression of the basic *PR1b1* gene but have no effect on the expression of the acidic *PR1a2* counterpart.

Table 2. GUS activity induced by different chemicals in transgenic tobacco plants harboring promoter-GUS constructs

Construct	Treatment ^a					
	Buffer	SA	ACC	Ethephon	H_3PO_3	HCl
(–914) <i>PR1b1/GUS</i>	400	28,400	18,000	26,000	1,060	1,200
(–418) <i>PR1b1/GUS</i>	112	3,360	940	1,050	330	NT
(–1,600) <i>PR1a2/GUS</i>	3,700	3,500	3,500	4,080	2,900	3,200
(–334) <i>PR1a2/GUS</i>	3,010	3,060	3,200	3,331	NT	NT

^a Leaf disks from transgenic plants were assayed for GUS activity after flotation for 48 h on buffer solution, buffer solution containing 0.5 mM salicylate (SA), 0.1 mM 1-aminocyclopropane-1-carboxylate (ACC), 0.5 mM ethephon, 1 mM phosphonic acid (H_3PO_3), 1 mM hydrochloric acid (HCl). For each construct, the average GUS activity in two F_1 plants derived from two independent primary transformants is given. NT = not tested.

DISCUSSION

Here we describe the characterization of two closely related genes from tomato plants encoding basic (*PR1b1*) and acidic (*PR1a2*) members of the pathogenesis-related PR-1 family. Comparison of the *PR1b1* and *PR1a2* nucleotide and amino acid sequences reveals remarkable homology all along the coding regions (Figs. 1 and 2) except in the C-terminal portion, in which the *PR1a2* isoform contains an extra tail of nine amino acids. This short C-terminal motif may determine a cellular localization of *PR1a2* (presumably extracellular) different from that of *PR1b1* (presumably vacuolar), as has been suggested for other PR isoforms (Neuhaus et al. 1991). This high homology extends even outside the ORF, either toward the 3' end or the 5' promoter region (Figs. 1 and 2A), and thus it seems likely that the two genes arose through a recent gene duplication event.

In spite of this strong conservation, the two genes have highly divergent expression patterns in transgenic tobacco plants using chimeric genes constructed by fusion of their promoter regions to the GUS reporter gene. Transformants containing the GUS gene under the control of the full and a deleted 5' promoter region of the acidic *PR1a2* gene showed no inducibility by TMV infection or pathogenic signals such as SA or ethylene but rather demonstrated a reproducible transcriptional activation which appears to be governed by developmental cues (Fig. 4). On the other hand, parallel analysis of transgenic plants containing the *PR1b1* promoter-GUS fusion did not reveal any detectable constitutive expression. This indicates that, unlike the acidic *PR-1* gene, the one encoding the basic isoform is not under developmental regulation.

The confined transcriptional activation of the *PR1a2* gene in the above-mentioned tissues constitutes an unexpected example of a precise mechanism of constitutive expression of a PR gene. How such a cell-type-specific expression pattern has evolved from common ancestral and pathogen-inducible genes, or vice versa, and how its expression is interpreted by the healthy plants remains unknown. Other defense-related genes also show constitutive expression and are under developmental regulation (Lotan et al. 1989; Zhu et al. 1993; Constabel et al. 1995; Liang et al. 1989). However, these differ from *PR1a2* in being induced by pathogenic signals in previously silent tissues.

Although we cannot disregard the possibility that the encoded acidic *PR1a2* protein may have a nondefensive metabolic function, we may speculate that the expression of this gene may be related to its participation in a certain type of immunity and that this mode of gene regulation may be the basis of why plants show natural resistance to certain types of pathogens. This argument may gain acceptance if we consider that transgenic plants constitutively expressing the homolog PR-1a isoform gene from tobacco under the control of the 35S promoter have increased tolerance to the fungal pathogens *Phytophthora parasitica* and *Peronospora tabacina* (Alexander et al. 1993), implying that *PR-1* genes play a role in disease resistance. Thus, it appears as if plants may have evolved a complementing set of both constitutive and inducible *PR-1* genes, which may be the basis for an explanation of the broad-spectrum immunity of plants to most pathogens.

At variance with *PR1a2*, the *PR1b1* gene shows a reproducible pathogen-mediated inducibility, as observed after TMV

inoculation (Fig. 5). This induction occurred in the tissues immediately surrounding necrotic lesions and concurs with the reported accumulation of endogenous PR-1 proteins from tobacco (Antoniw and White 1986). Conversely, while endogenous tobacco genes (referred to as SAR genes) are concomitantly induced in upper, uninoculated leaves upon TMV infection (Brederode et al. 1991; Ward et al. 1991; Lawton et al. 1993), the *PR1b1* gene under consideration is not.

Regulatory elements controlling pathogen induction of genes encoding PR proteins have been characterized in a number of cases; however, they are widely different, making it difficult to define a minimal promoter necessary for pathogen induction of different PR genes. A well-conserved DNA motif has been found to be present in a number of genes encoding basic isoforms of PR proteins (Meller et al. 1993; Alonso et al. 1995; Eyal et al. 1993 and references therein). In these genes, the GCCGCCTC sequence motif is present and appears to be necessary for local induction of gene expression in an ethylene-dependent manner. This same motif is present in the tomato *PR1b1* and is thus a potential *cis*-acting element for the regulation of expression of this gene member. In fact, transgenic plants harboring a 5' deletion in the *PR1b1* promoter (to -418), which eliminates this 8-bp motif, present a drastic reduction of expression upon TMV infection, suggesting that this conserved DNA motif may also be necessary for full expression of *PR1b1*, as has been deduced for other PR genes encoding proteins with basic pI. Since the GCCGCCTC motif has been shown to mediate ethylene activation, it is tempting to speculate that the induction of *PR1b1* is, at least partly, controlled by the level of endogenous ethylene produced by TMV infection. This gains support if we consider that ethylene has long been known to be produced when cells undergo necrosis resulting from pathogen infection (Ross and Williamson 1951; De Laat and Van Loon 1982, 1983) and is consistent with the fact that genes encoding ethylene-biosynthetic enzymes are induced locally as a result of TMV infection (Knoester et al. 1995). Similarly, the absence of TMV-induced expression of the genes for ethylene biosynthesis in the uninoculated, systemic leaves (Knoester et al. 1995) corresponds to the absence of TMV-induced gene expression for *PR1b1*/GUS in these leaves (Table 1). Furthermore, the *PR1b1* gene is strongly induced by low concentrations of the ethylene precursor ACC or by the ethylene-releasing ethephon (Fig. 5), and mRNA accumulation for this gene in tomato plants is suppressed by inhibitors of ethylene action (Tornerio et al. 1994). All this points toward a role for ethylene in the local expression of PR genes with a basic pI, or at least some members of this subclass, during the HR. However, this *PR1b1* gene is also inducible by SA, and this finding indirectly supports the notion that different signaling processes may operate to set in motion the expression of basic and acidic PR genes.

A variety of mutants displaying increased resistance to pathogen attack have been described (for a recent review, see Hunt and Ryals [1996]). In these mutants, SAR responses are activated in the absence of pathogen infection and always contain elevated levels of SA and increased expression of SAR marker genes (Greenberg and Ausubel 1993; Dietrich et al. 1994; Bowling et al. 1994). Additionally, the signal transduction mediating SAR has been demonstrated to be ethylene-independent in *Arabidopsis* (Lawton et al. 1994). Furthermore, SA applied to plant tissue has been shown not to be converted

to ethylene (Van Loon and Antoniw 1982), while local application of the ethylene precursor ACC or ethephon to plant tissues (J. Fallos et al., *unpublished results*) provokes rapid and long-lasting increases in SA. All these observations support the hypothesis that there are at least two classes of signal transduction pathways activated during incompatible plant-pathogen interactions and that differentially set in motion expression of *PR* genes and presumably other defense responses. One is activated locally and involves ethylene as a transient pathogenic signal that coexists with or can signal the enhancing of SA. This pathway appears to be able to activate local defenses (e.g., basic isoforms of *PR* proteins) but is independent of SAR or not associated with it. The other signaling process, which appears to be independent of ethylene but mediated by SA, is associated with SAR in a long-lasting way and is accompanied by the expression of acidic *PR* members, but not basic ones, in distant tissues.

In support of this duality are the recent results of Beffa et al. (1996), who demonstrated two different classes of signaling pathways, mediated by G-proteins, in transgenic plants expressing the cholera toxin (CTX). One class, which is insensitive to CTX, is associated with HR and is required for induction of class I *PR*-2 and *PR*-3 proteins by ethylene. The second class, which is activated by CTX, is required for SAR-associated induction of acidic *PR*-1 and class II *PR*-2 and *PR*-3 proteins. Furthermore, in an attempt to genetically dissect the signal transduction regulating defense responses in *Arabidopsis*, Glazebrook et al. (1996) have shown that *eds5*, which is an enhanced disease susceptibility mutant and allelic to mutant *npr1*, a nonexpresser of *PR* genes (Cao et al. 1994), does not induce *PR*-1 in response to SA but shows induced expression of *PR*-1 upon infection with *Pseudomonas syringae* (Psm ES4326). This observation can be explained as if induction of *PR*-1 expression in response to bacterial infection results from the combined effect of two signaling pathways, one that is mediated by SA through *NPR1* and one that is *NPR1*-independent and may involve ethylene as a local inducer. The existence of more than one signaling pathway is further supported by the observation of Green and Fluhr (1995) that the UV-B induction of an SAR marker gene, *PR*-1, is independent of SA action but mediated by active oxygen species which could be originated through the activation of a parallel signaling process.

How these different signaling processes are interlinked and how they operate to set in motion different batteries of plant defenses, either locally or in distant tissues, in such a precise and coordinated fashion remains enigmatic. Comparative studies of different plant-pathogen interactions using either constitutive, HR-, or SAR-specific *PR* promoters as molecular tools will help address these questions in future experiments.

MATERIALS AND METHODS

Plant material and growth conditions.

All plants were grown at 22°C in growth chambers programmed for a cycle of 14 h of light and 10 h of darkness.

Library screening and DNA sequence analysis.

Library screening using radiolabeled cDNA was performed as previously described (Tornero et al. 1994). A tomato genomic DNA library constructed in λ -EMBL3 (Clontech) was

screened with a radiolabeled pTE28.1 cDNA probe (Tornero et al. 1993), and the positive clones were isolated and purified by standard techniques (Maniatis et al. 1982). DNA sequencing and computer-assisted comparison of DNA sequences were performed as described before (Tornero et al. 1994).

Promoter-GUS fusions, plant transformation, and analysis of transgenic plants.

Oligonucleotides o17.2 (5'-GGGATCCTTTAGGTTTAGT-GCAG-3') and oR3-1 (5'-GGGATCCGCTTTGAGAGAA-AAT-3') served as primers for the incorporation of synthetic *Bam*HI restriction sites in the *PR1b1* and *PR1a2* upstream sequences, respectively, by site-directed mutagenesis (Kunkel et al. 1987). The synthetic *Bam*HI site was introduced into the promoters upstream regions at positions -1 relative to the translation initiation site. A blunt-ended *Bam*HI fragment containing *PR1b1* promoter sequences -914 to +56 relative to the transcription start site was cloned upstream of the β -glucuronidase gene in pBI101.1 (Jefferson et al. 1987). An *Xba*I-*Bam*HI fragment containing *PR1a2* promoter sequences -1,600 to +47 was similarly cloned in pBI101.1. Promoter deletions were carried out by using internal restriction sites (*Hind*III). The resulting fusions were verified by nucleotide sequence analysis using primers specific for the promoter region of each gene. The resulting plasmids were then mobilized into *Agrobacterium tumefaciens* LBA 4044 as described by Bevan (1984). Leaf disks of *N. tabacum* cv. Xanthi-NC were transformed as described by Horsch et al. (1984). Primary transformants were allowed to self-fertilize, and F₁ seeds were collected and germinated on MS agar medium containing kanamycin for selection. Transgenic seedlings were then transferred to soil. Transformants were assayed for GUS activity by a fluorometric assay or by an in situ assay using the colorimetric substrate X-Gluc (Jefferson 1987). For each construct, expression was determined in at least five independent transgenic lines.

Induction of the *PR1a2/GUS* and *PR1b1/GUS* genes.

Seven- to 8-week-old plants were used for protein extraction and GUS assays following treatment with chemicals or inoculation with TMV. For induction with SA, ethephon, acid derivatives, or ACC, 1.5-cm disks were punched from leaves of transgenic plants and floated in a petri dish on 30 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 6.9) containing these chemicals at the indicated concentrations for 48 h at 22°C under a cycle of 14 h of light and 8 h of darkness. After incubation, leaf disks were directly analyzed for GUS expression or were frozen and stored at -70°C. For inoculation with TMV, leaves of plants were dusted with Carborundum and rubbed with a suspension of TMV strain U1 (1 μ g/ml) (provided by I. García, CIB, Spain) in 50 mM phosphate buffer, pH 7.2. Leaf disks were taken from inoculated leaves at 0, 1, 2, 3, and 4 days after inoculation and from upper, uninoculated leaves at 4, 6, and 8 days after inoculation.

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