

Pathogen, salicylic acid and developmental dependent expression of a β -1,3-glucanase/GUS gene fusion in transgenic tobacco plants

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Summary

The 5' flanking region of a gene encoding an acidic β -1,3-glucanase from *Nicotiana tabacum* was isolated and characterized. A chimeric gene composed of 1759 bp of the promoter sequence from the PR-2 gene was fused to the β -glucuronidase (GUS) coding region and used to transform tobacco. Transcriptional activation of the PR-2 promoter was investigated in response to inoculation with tobacco mosaic virus (TMV), after treatment of leaves with salicylic acid (SA), and in specific tissues during the normal development of healthy plants. In TMV-inoculated transgenic plants, GUS activity was induced locally around necrotic viral lesions and systemically in uninoculated leaves. GUS activity was also induced by treatment of leaves with SA. The chimeric gene was expressed in floral organs of healthy plants and in newly germinated seedlings. Analyses of a series of 5' deletions of the glucanase promoter indicated that the *cis*-acting elements necessary for induction by all these signals are localized in the region between –321 bp and –607 bp upstream of the transcription start site.

Introduction

Plants have developed several defense mechanisms that enable them to respond to pathogens and stresses. One common form of resistance to disease is the hypersensitive response (HR), which is characterized by formation of a necrotic lesion at the site of pathogen entry and restriction of pathogen growth and spread. Accompanying the HR is the *de novo* synthesis of a large number of novel proteins with putative roles in defense. A subset of these proteins

is known as pathogenesis-related (PR) proteins. The PR proteins are classified into five or more unrelated families (for review see Cutt and Klessig, 1992; Linthorst, 1991). For most of these families, differentially regulated genes encoding acidic and basic isoforms have been reported (Memelink *et al.*, 1990; Ward *et al.*, 1991b).

Several studies suggest that plant hydrolytic enzymes, such as β -1,3-glucanases and chitinases that belong to the PR-2 and PR-3 families, respectively, may be components of a general defense system against pathogen invasion in several different plant species (Benhamou *et al.*, 1989; Jondel *et al.*, 1989; Joosten and De Wit, 1989; Kauffman *et al.*, 1987; Kombrink and Hahlbrock, 1986; Kombrink *et al.*, 1988; Meins and Ahl, 1989; Vogeli *et al.*, 1988). *In vitro* studies (Mauch *et al.*, 1988) have demonstrated that glucanases and chitinases act synergistically to inhibit fungal growth. In addition, glucanases can release short glycosides from both host and pathogen cell walls that can act as elicitors to enhance host defense responses (Hahn *et al.*, 1988).

However, direct tests of the role of these hydrolytic enzymes to protect transgenic plants have produced inconclusive results. Broglie *et al.* (1991) found increased resistance against infection by *Rhizoctonia solani* in transgenic tobacco plants which constitutively expressed a bean chitinase gene. However, another study (Neuhaus *et al.*, 1991) indicated that constitutive expression of a tobacco basic chitinase gene did not protect transgenic *Nicotiana sylvestris* plants against infection with the fungus *Cercospora nicotianae*. Also, inhibition of the synthesis of basic glucanases using anti-sense RNA in transgenic *N. sylvestris* did not alter the susceptibility of these plants to this fungal pathogen (Neuhaus *et al.*, 1992).

There is now evidence, based principally on amino acid homology, for at least three distinct classes of glucanase genes in tobacco. These encode: the class I basic, vacuolar-targeted proteins which undergo substantial post-translational modifications including removal of a carboxyl-terminal extension (Shinshi *et al.*, 1988; Van den Bulcke *et al.*, 1989); the class IIa acidic, extracellular proteins which include the PR-2a (2), PR-2b (N) and PR-2c (O) proteins (Ward *et al.*, 1991a); the class IIb neutral or basic glucanases; GL153 and GL161, closely related by sequence to the class IIa glucanases but containing carboxyl-terminal extensions (Ward *et al.*, 1991a); the class IIc stylar-specific, extracellular glucanase Sp41, related to classes IIa and IIb (Ori *et al.*, 1990); and the class III acidic PR-Q' glucanase, a tobacco homolog of the elicitor-releasing glucanase from soybean

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(Payne *et al.*, 1990). The class I, IIa, IIb and III glucanase genes are induced by pathogenic stress; thus, these genes are considered PR genes.

The PR-2 genes are expressed during the hypersensitive (resistance) response in TMV-inoculated leaves (Côté *et al.*, 1991; Linthorst *et al.*, 1990; Ward *et al.*, 1991b). In uninoculated leaves, their expression correlates with the development of systemic acquired resistance (SAR). The PR-2 genes are also induced by treatment with salicylic acid (SA), a putative endogenous signal for activation of disease resistance (Malamy *et al.*, 1990, 1992) and other chemical inducers (Côté *et al.*, 1991; Ward *et al.*, 1991b). The class I basic, vacuolar glucanases are constitutively expressed in healthy *Nicotiana* plants. A gradient of expression of these genes has been observed in plants from bottom to top with the highest level in the roots (Castresana *et al.*, 1990; Neale *et al.*, 1990). The class I glucanase genes are expressed in many tissues and are regulated by phytohormones such as auxin, cytokinin and ethylene (De Loose *et al.*, 1988; Felix and Meins, 1987; Memelink *et al.*, 1990) and by stresses such as wounding and UV irradiation (Brederode *et al.*, 1991). Expression of the various classes of glucanase genes is associated with physiological processes such as flower development (Neale *et al.*, 1990; Ori *et al.*, 1990), fruit maturation (Hinton and Pressey, 1980), regulation of phloem transport (Wong and MacLachlan, 1980) and seed germination (Høj *et al.*, 1989). However, little is known about the regulatory sequences which control the expression of these genes.

Here we report the characterization of the promoter region of a gene very closely related to the PR-2c gene which encodes an acidic, extracellular glucanase. We analyzed the expression of this PR-2 promoter/*uidA* (GUS) fusion gene after TMV inoculation or SA treatment in transgenic tobacco plants. In addition, these plants were used to study organ-specific and developmental regulation of the PR-2 gene promoter.

Results

Isolation and characterization of the promoter region for an acidic glucanase gene

We previously isolated a partial cDNA clone (designated λ FJ1) of the PR-2c glucanase gene of tobacco and used it to demonstrate that expression of the class II acidic glucanase genes was induced at both the mRNA and protein levels in tobacco inoculated with TMV or treated with chemical inducers (Côté *et al.*, 1991). To better understand the mechanism of induction we attempted to isolate the promoter of the corresponding PR-2 gene utilizing the inverse polymerase chain reaction (IPCR, Ochman *et al.*, 1988).

Examination of λ FJ1 plus several other glucanase cDNA clones obtained from our library indicated that the glucanase gene contained an internal *Eco*RI site (unpublished observations). Southern blot hybridization of *Eco*RI digested genomic DNA from *N. tabacum* cv. Xanthi nc using λ FJ1 as a hybridization probe revealed several intensely hybridizing bands including 2.4 and 2.8 kb fragments (Côté *et al.*, 1991). Because clone λ FJ1 is small (436 bp) and contains only sequences 5' of the internal *Eco*RI site, it appeared likely that the 2.4 and/or 2.8 kb fragments would be comprised primarily of sequences corresponding to the 5' flanking region of the glucanase coding region.

Utilizing the cloning scheme outlined in Figure 1 and described in Experimental procedures, we were successful in amplifying and cloning the band corresponding to the 2.4 kb *Eco*RI fragment; no amplification product was detected corresponding to the 2.8 kb band. Because terminal *Eco*RI sites were also generated by the primers used in the PCR reaction, approximately 200 bp of sequence corresponding to the distance from the internal genomic *Eco*RI site and the engineered *Eco*RI site were lost upon subcloning the fragment into a plasmid vector (Figure 1). The resulting 2.2 kb restriction fragment in pUC119 was designated pGluc-2.

Sequence analysis indicated that pGluc-2 contained a 219 bp open reading frame (ORF) corresponding to the 5' coding region of a PR-2 gene and 1759 bp of the 5' flanking region (Figure 2). The coding region of pGluc-2 is interrupted between the first and second guanidine of the codon for Gly₂₈ by a putative 216 bp intron with AG/GT and AG/GG consensus splice sites (Shapiro and Senapathy, 1987). The ORF sequence shares 94.6% identity to the PR-2c cDNA clone λ FJ1 (Côté *et al.*, 1991) versus 92% and 91% identity to the PR-2a and GL153 cDNA clones reported by Ward *et al.* (1991a). Although the pGluc-2 coding region exhibits 92% identity to the coding region of a genomic clone gl19 encoding the acidic glucanase PR-2b (Linthorst *et al.*, 1990), the introns of both genes share only 55% homology. While the 5' flanking regions of the gl19 and pGluc-2 clones exhibit 63.5% identity overall, the sequences between the translation initiation codon and position -72 from transcription start site (see next section) are 92% identical. pGluc-2 shows less identity (40–50%) in the coding region and little, if any, in the noncoding region to the basic glucanase genes (Castresana *et al.*, 1990; Shinshi *et al.*, 1988). In summary, while the pGluc-2 genomic clone most closely resembles the λ FJ1 clone (PR-2c gene), it appears to be a distinct gene. Thus, we will refer to it as the PR-2d gene.

The transcriptional start site for the PR-2d gene was determined using primer extension analysis. RNA prepared from leaves of plants treated with 1 mM SA or H₂O (control) for 36 h was hybridized to an oligonucleotide

complementary to the coding strand of the PR-2d (and PR-2c) gene (see Experimental procedure for details). After extension with reverse transcriptase, two products differing in length by one nucleotide were observed using

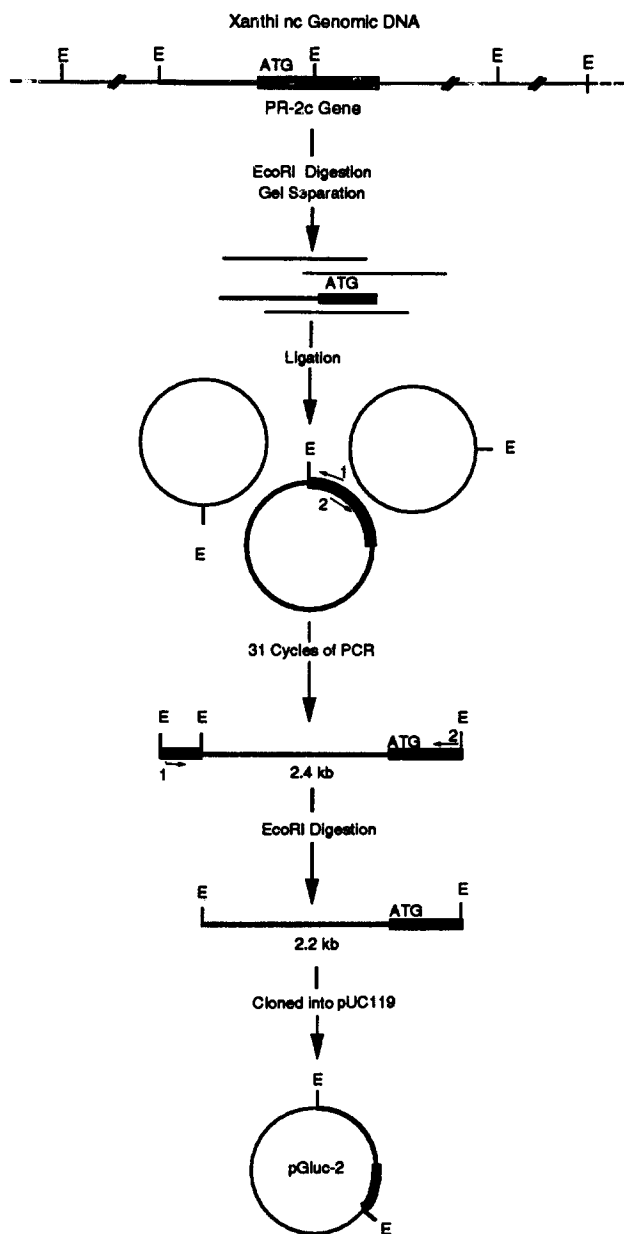


Figure 1. Schematic of IPCR based strategy for cloning a tobacco glucanase promoter.

Total tobacco genomic DNA was digested with *EcoRI* and fractionated on a 1.0% agarose gel. DNA from 2.2 to 2.9 kb was extracted from the gel, self-ligated, and subjected to 31 cycles of PCR using primers 1 and 2 (arrows). The amplification products, including a major 2.4 kb fragment, were digested with *EcoRI*, and the corresponding 2.2kb fragment was isolated and cloned into pUC119. The resulting plasmid was designated pGluc-2. The coding region of the PR-2c (or PR-2d) is represented by the thick bar while the plant genomic sequences are indicated by heavy lines, and the vector sequence is denoted by a thin line. *EcoRI* restriction sites (E) are indicated as is the putative promoter region and initiating ATG.

the RNA from SA-treated plants; no extension products were detected in H₂O-treated plants (Figure 3). The calculated lengths of the 5' untranslated leaders on these two mRNA species were 52 and 53 nt. The start site of the longer leader is designated +1 in Figure 2. A putative TATA box at -25 and CAAT box at -76 are indicated in Figure 2.

Pathogen- and SA-induced expression of a chimeric PR-2d/GUS gene in transgenic tobacco plants

To analyze PR-2d gene regulation, a chimeric PR-2/GUS reporter gene was constructed and introduced into tobacco. The chimeric PR-2d(-1706)/GUS gene was constructed by fusion of the upstream promoter region of the PR-2d gene (-1706 to +51) to the ATG initiation codon of the GUS coding sequences and the 3' nopaline synthase (NOS) regulatory sequences. An *EcoRI*-*HindIII* fragment containing this expression cassette was cloned into the binary vector pGA482 (An, 1986) and then introduced into *N. tabacum* cv. Xanthi nc by *Agrobacterium tumefaciens*-mediated leaf disc transformation.

To assure that transcription from the chimeric PR-2d (-1706)/GUS gene initiated at the same site(s) as the endogenous gene, primer extension analysis was done with an oligonucleotide complementary to the coding strand of the GUS gene. Identical transcriptional start sites were found as for the endogenous PR-2d gene (data not shown).

In 12 of the 15 independent kanamycin resistant (Kan^R) transformants, the chimeric gene was activated during the hypersensitive response to TMV infection. GUS activity was detected histochemically by staining leaves with 5-bromo-4-chloro-3-indolyl- β -glucuronide (X-Gluc) 2 days after inoculation with TMV. The chimeric gene driven by PR-2d promoter was expressed at high levels in the tissue immediately surrounding the local lesion as indicated by distinct blue staining around each lesion at 40 h post-inoculation (Figure 4a). At 72 h post-inoculation when necrotic lesions were fully developed, GUS activity was detected at greater distances from the lesions and along veins which passed near or through lesions (Figure 4b). No GUS activity could be detected before inoculation or after mock inoculation. In contrast, GUS activity was detected throughout the leaf in control plants carrying a CaMV 35S/GUS reporter gene (described in Experimental procedures), consistent with earlier observations that expression from the CaMV 35S promoter is constitutive and relatively non-tissue specific.

Previous studies have shown that after TMV inoculation of the lower leaves of *N. tabacum* cv. Xanthi nc, the acidic glucanases are induced in the virus-free, uninoculated, upper leaves, as well as in the primary inoculated leaves (Côté *et al.*, 1991). To investigate the systemic induction of the PR-2 promoter by viral infection, GUS activity was determined in three independent transgenic lines contain-

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-1706  GAATTCTGAACCTTAGTTATGTGTTCAAAGCAGGATCTGAGGGGTTAGGGTATGATTCGAC
      ACTATTGCATAAAAGTTGTAAACTTATGAGTTCATAAATTTGATTTGAACTTTAATTTTG
      AGTTGCGATGTTTCTATATATGTTTTGAACCCTTGAACAAGTTCGATTTGAATATTTAGA
      CAAGATTCAAAGTCAAATTTGGACTCGACTAAAATATCAAGATGTATTTCCACTTGGATG
      TTTATCTAGATTCATGTCCCTCCGTGAGATTTGACAAAGTCAGACTTCTTAGCTATAATTA
-1406  ATTAATTGTATTCTTTTGCCTGGAAAACTATAACACCCAATGCAAGACAAAAGTTTGT
      AATTCCTCGGTCTTAATTTATATAGCAGTAATAGATTTCGGCATTNTTAAAATTTATGATC
      TAAAATAAGCCATATATGTTTGCATAATTATAAACCATCTTATTAAGATAAAAATGAGAAG
      TTTAAAGTTAAATTTATTTTAAATATAAAAGTATGTCATTCTTTTTTAGATAAGCTAATT
      AAAGAAAAGTGTATCGGATAAAGTGGGACAAAGGAATATCAAGATTAATTAATCGATCTA
-1106  CTTTTAAAATCAAGTATCACCTTAGCTCAATAAGAAAAAAAAAATCTTGTTTTGGTGTA
      GTAATTAATTTGGTATTTGTCTAAACTCCTCTTCTCTCTTAAATACAACGATAATACT
      AATTGAGATCCCACAAGTGGGGTTCGAGAAGAGTAGGGTGTACGTAGACTTTACCCCTAC
      CTTTTGTGAGATAGAGAGGCTGGTCTGATAGAGCCTCGACTTAAAAAAACTTTTTCAA
      AAGAGGTTTTAAAATTAAGAGAGTAAAAAAGAAGAAGATGAAAATACTAATAAGAAAA
-806  AAGAAAAATATTGATGATGATGATAATAGTAGTAGTAGGCGCTTTTTAAAACCAATA
      GTCACCTTTTTGAATTTAAAAAAGAAAACCTATTAAAAACGTTTTCTGAAATTTAATTTAA
      AAATGTTTTAAACTAATTAATAAAATAATAGGTGACTAAAAATTAATAATTACATCTAA
      AATATTCTTTAAAATATTCAAACCTATATAAGACAAAGAAGTGCTTTTTAAACGCTTCTTC
      CTTTCTTCTTTTTTCCAACGAGGGATAAAACAACCTTTTCTAAATACCTCCATCAACTAA
-506  TCTAACAAATTTTGCATCTATCACTACCAATTAATTTTGGAAAAGTGACAAAATTTACCC
      CGATGAACTTTTGTAGTATAGTTTCAAGATTACAAGCTAGAAAAATAATAGTATTTAAATTA
      AGTCACGAAATGCTCAATCTCGCATGCCGTATAGTATAGGGGCAGCTTCGACCTCCTTC
      TCCGAATATTCGACCATCTTTTTCTCGTAAAATCAAACGCTCTCCATCACTGTACTAGATT
      TGAACCTTCTACATTTTTTTTTCCACTGTGACATAAAATACACTCTCTATACTTGGTATG
-206  ACTTGGCTTCACTGGGCTTGACTTGGTTATTAACCTACTTAATACACTGTCTATATTCC
      ATGACGTGCTTCACTGGGTATGACTTGGTTAATAAAATTACACTATTCTTTAACTGCTT
      CACAGGGCAATGGACTTTGGCTTTTTAAGTCTTTGTAGGTCTTCTTCTACTTGTCTATAT
      AAGAAGCAGCCTAATGGTTCCTGAAACGCACAATTTAGCTCAAGTGTATCTAATTATTC
      TCTCATTTCATTTTAGCTATGGCTTTATGCATTAATAAATGGCTTTCTTGCAGCTGCCCT
      M A L C I K N G F L A A A L
95    TGTACTTGTGGATTGTTAAGGTGCAGTATCAAATGATAGgtctctctctcaccacac
      V L V G L L R C S I Q M I G
      acacatgtacactacatgtatatgcacctgtacgatgcgatcaacttatatacactaa
      cactgagttatccaagagggataggatcacttcccaattattaaatTTTTTTcttc
      tttcttagtagtgcacctatatactaaaaatcctagatccggctctgttcttatgtatt
      tttttcttatcatgcagGGGCACAATCTATTGGAGTATGCTATGGAAAAATTGCCAACAA
      A Q S I G V C Y G K I A N N
      TTTACCATCAGATCAAGATGTTATAAACTATAACAATGCTAATGGCATCAGGAGGATGAG
      L P S D Q D V I K L Y N A N G I R R M R
455  AATATACTATCCAGAAACAATGTCTTCAATGCT
      I Y Y P E T N V F N A
  
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Figure 2. Partial nucleotide sequence of the *N. tabacum* PR-2d gene. The deduced amino acid sequence of the amino terminus of the PR-2d ORF is given below the DNA sequence. A 216 bp intron (lower case letters) interrupts the coding sequence. The transcriptional start sites determined by primer extension analysis are indicated by dots (•). The 5' most start site is denoted +1. Putative CAAT and TATA boxes are underlined. The dashed line represents the sequence complementary to Primer 1 used for PCR amplification.

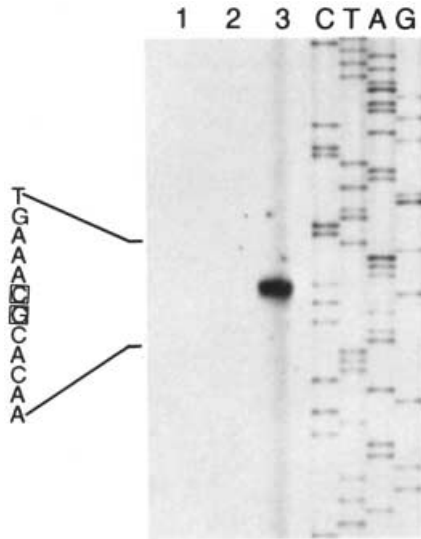


Figure 3. Primer extension mapping of the PR-2d gene transcriptional start sites. Twenty micrograms of total RNA isolated from leaves treated with H₂O (lane 2) or SA (lane 3) was annealed to a 5'-end-labeled oligonucleotide specific to the PR-2c and PR-2d genes. Lane 1 represents the negative control sample without RNA. Products of the extension reaction were separated on a 6% polyacrylamide gel. Location of the transcriptional start sites was determined based on the comigration of the primer extension products relative to the sequence ladder of the corresponding 5' untranslated region.

Table 1. Local and systemic induction of GUS activity in PR-2d(-1706)/GUS transgenic plants after TMV inoculation

Days post-inoculation	Gus activity		
	Mock inoculated	TMV	
		Inoculated leaves	Uninoculated leaves
0	120	60	82
2	37	3110	75
4	52	15609	880
6	185	nt ^b	4536
8	87	nt	8496

^aGUS activity was determined in F₁ progeny of three independent PR-2d(-1706)/GUS transformants at 0, 2, 4, 6 and 8 days post mock or TMV inoculation. The data represent the average GUS activity observed in the three plants at each time point. GUS activity in untransformed control plants in the absence of TMV inoculation was 6.1. Specific activity was expressed as pmol 4-MU min⁻¹ mg protein⁻¹.

^bnt, not tested.

ing a single locus of the transgene after mock or TMV inoculation. Two inoculated and two upper, uninoculated leaves from each plant were collected at various times after TMV inoculation (Table 1). Only very low levels of GUS activity were detected in inoculated leaves immediately following infection with TMV or in mock-inoculated

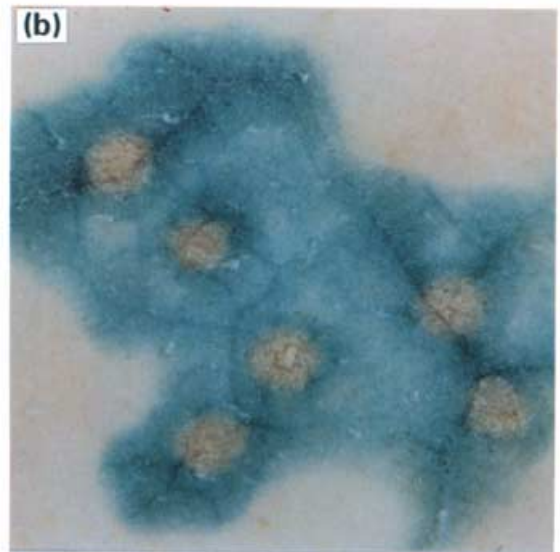
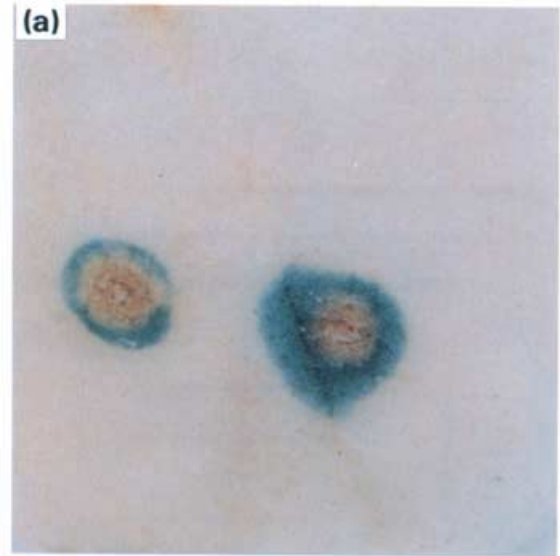
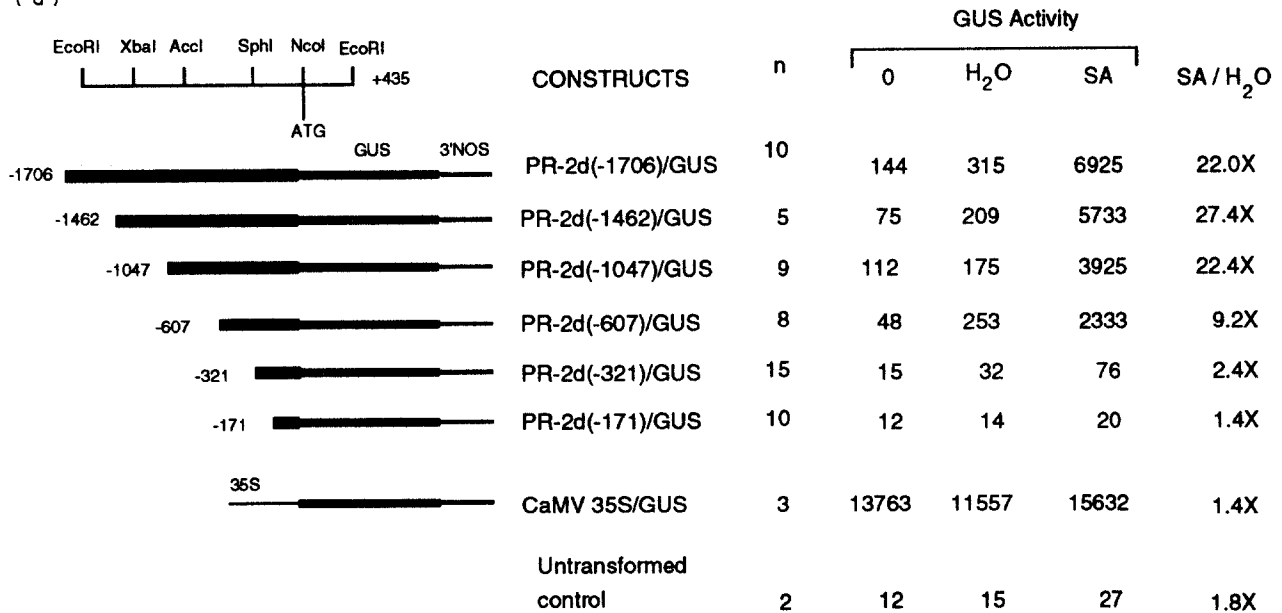


Figure 4. Histochemical localization of GUS activity during the hypersensitive response. Leaf discs were punched out of leaves of primary transformants carrying the PR-2d(-1706)/GUS construct at 40 h (a) and 72 h (b) after inoculation. After vacuum infiltration with an X-Gluc solution, they were incubated at 37°C for 6 h to allow development of the blue color corresponding to GUS activity. Chlorophyll was removed with ethanol washes to facilitate detection of the blue stain.

plants throughout the experiment. In contrast, by 2 and 4 days post-inoculation (dpi) there was a 50- to 150-fold induction of GUS activity in TMV-inoculated leaves. Low levels of GUS activity were detected in upper, uninoculated leaves as early as 4 dpi. By 6 and 8 dpi levels of GUS activity in uninoculated leaves rose substantially and were comparable with the levels detected in inoculated leaves 2-4 dpi. The class IIa glucanase genes are induced by SA

(a)



(b)

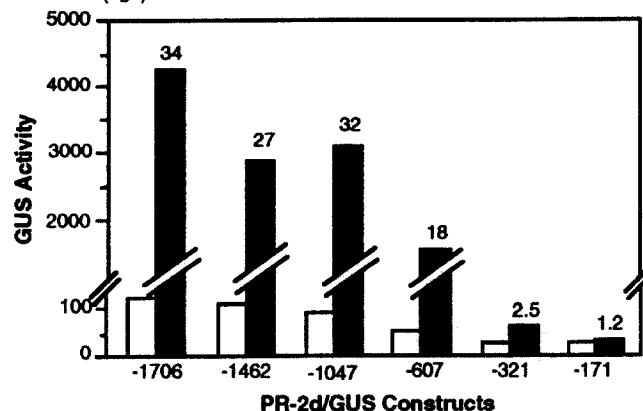


Figure 5. Structure and expression PR-2d/GUS chimeric genes in transgenic tobacco.

The EcoRI–NcoI fragment (–1706 to +51) from the pGluc-2 and deletion mutants in 5' flanking region of the PR-2d gene were fused to the GUS coding region which was linked to the nopaline synthase 3' (3'NOS) regulatory sequences. The PR-2d promoter sequences in each construct are represented by the thick bar while the GUS and 3' NOS regions are depicted by progressively thinner lines. The base pair coordinates at the left indicate the length of promoter fragment with respect to the PR-2d transcriptional start site.

(a) GUS activities (pmol 4-MU min⁻¹ mg protein⁻¹) are expressed as averages obtained with F₁ progeny from *n* number of independent transgenic plants which carry a single copy of the construct. GUS activity was assayed in leaf discs taken before treatment (0 time) or after floatation for 48 h on H₂O or H₂O containing 1 mM salicylic acid (SA). Controls were untransformed tobacco (*Xanthi nc*) and plants containing CaMV 35S promoter (–800 to +4) fused to GUS/3'NOS. The right-hand column shows the ratio of promoter activation by SA versus water.

(b) GUS activity was measured in leaves of F₁ progeny from three to six independent transgenic lines for PR-2d(–1706)/GUS, PR-2d(–607)/GUS, PR-2d(–321)/GUS and PR-2d(–171)/GUS 3 days after mock (open bars) or TMV (dark bars) inoculation. The data for PR-2d(–1462)/GUS and PR-2d(–1047)/GUS are for two (averages) and one independent transgenic lines, respectively. The numbers under the bars indicate the length of the PR-2d 5' sequences. The induction factors by TMV inoculation relative to mock inoculation are indicated above the respective bars. The induction factor for untransformed control plants was 1.9.

(Côté *et al.*, 1991), a putative endogenous signal for activation of disease defense response (Malamy *et al.*, 1990, 1992). We found that application of exogenous SA to the transgenic tobacco plants also activated the chimeric PR-2d(–1706)/GUS gene leading to a large increase in the level of GUS activity in treated plants (data presented below). Thus, regulation of the PR-2d promoter in this

chimeric gene appears to be similar to the endogenous PR-2 genes that are induced locally and systemically after infection with TMV or locally after treatment with SA (Côté *et al.*, 1991; Ward *et al.*, 1991b). Moreover, this implies that expression of the corresponding endogenous glucanase gene is controlled to a significant extent at the transcriptional level.

Identification of PR-2d promoter elements required for TMV and SA induction

To define the region necessary for regulation of PR-2 gene expression, a series of 5' deletions in the PR-2d regulatory region were constructed. Deletions starting from -1706 and ending at positions -1462, -1047, -607, -321 and -171 were fused to the GUS coding and 3' NOS regulatory sequences (Figure 5a) and introduced into *Xanthi* plants. For each promoter construct, 8–15 independent transformants were isolated and self-fertilized. To eliminate the effect of gene copy number on GUS activity, only single copy F₁ progeny of primary transformants were used for further studies. Copy number was determined by genomic Southern blot analysis and a Kan^R segregation test.

The F₁ progeny of the deletion series were first tested for induction by SA. GUS activity was measured in leaf discs of 6-week-old plants taken before treatment or after flotation for 48 h on H₂O or on a 1 mM SA solution. Figure 5a shows the average GUS activities detected in discs from plants carrying the different PR-2d promoter deletions. In leaf discs assayed immediately after punching, no significant GUS activity was observed (0 time sample). A two- to fivefold increase in GUS activity over background levels was observed after flotation on H₂O. Very low levels of expression and no significant induction of GUS activity by SA was observed in leaf discs from plants containing the PR-2d(-171)/GUS construct. Similarly, very low levels of GUS activity and little, if any, inducibility by SA was found with the PR-2d(-321)/GUS construct. In contrast, in PR-2d(-1706)/GUS, PR-2d(-1462)/GUS and PR-2d(-1047)/GUS transgenic plants, SA treatment increased GUS activity over 20-fold. A two- to threefold decrease in inducibility of GUS was detected in transgenic plants containing the PR-2d(-607)/GUS construct versus the full-length promoter construct. However, these plants still showed relatively high levels of inducible PR-2d/GUS expression. No significant changes in expression of the CaMV 35S/GUS gene fusion were observed in control transgenic plants treated with SA.

For each construct, F₁ progeny from one to six independent primary transformants were tested for inducibility by TMV infection. Three days after inoculation plants containing the PR-2d(-1706)/GUS, PR-2d(-1462)/GUS, PR-2d(-1047)/GUS and PR-2d(-607)/GUS constructs exhibited high levels of GUS activity (Figure 5b). In contrast, only slight, if any, SA-inducible increases in GUS activity were found in PR-2d(-321)/GUS and PR-2d(-171)/GUS transgenic plants compared with untransformed control plants. This pattern of induction was very similar to that observed in these same plants after SA treatment (Figure 5a). These results indicated that 607 bp of 5' flanking region of the PR-2d gene were sufficient for TMV- and SA-regulated expression.

Developmentally regulated expression of chimeric PR-2d/GUS gene

The expression of glucanase genes in tobacco is not only induced by pathogen assault and treatment with compounds that enhance resistance like SA or isonicotinic acid, but exhibits tissue-specific and developmental regulation (Côté *et al.*, 1991; Linthorst *et al.*, 1990; Van den Bulcke *et al.*, 1989; Ward *et al.*, 1991b). To obtain a better understanding of the mechanism and specificity of tissue-specific and developmental regulation, the patterns of expression of the chimeric PR-2d gene were analyzed in healthy transgenic plants using X-Gluc histological staining. In contrast to the previously reported expression patterns of the class I, basic glucanase genes, the PR-2d(-1706)/GUS gene (representative of the class IIa, acidic, extracellular genes) was not expressed in roots or in lower or upper stems and leaves of 12-week-old plants (data not shown). However, expression of the PR-2d(-1706)/GUS gene was detected in young seedlings (Figure 6d) and in developing floral organs (Figure 6a–c).

During early seedling growth (3–4 days post-planting), PR-2d(-1706)/GUS gene expression was detected in the root where it protrudes from the seed coat and in the cotyledons (Figure 6d). In the cotyledons, GUS activity was located in close proximity to the vascular system (Figure 6d). Activation of the PR-2d(-1706)/GUS gene fusion was also observed in different tissues during the development of flowers. At early stages before or during anthesis, GUS activity was detected at the base of the flower, where the pedicel joins the ovary (Figure 6a). The sepals, particularly the trichomes of the sepal, exhibited high levels of GUS activity (Figure 6b). At 1 week post-anthesis and later, GUS activity was lower but still detectable in the sepal. At these times, the major sites of PR-2d(-1706)/GUS activity were in the center of the placenta down into the pedicel (Figure 6c). No GUS activity was observed in the remaining tissues of the flower.

To determine the location of the regulatory element(s) in the PR-2d promoter responsible for this pattern of expression, the transgenic plants containing the different 5' deletion constructs were analyzed during floral development. Deletion of sequences upstream of -607 did not qualitatively affect tissue-specific expression. GUS activity was evident in all tissues (sepals, placenta, ovary) in transgenic plants carrying the PR-2d(-607)/GUS gene, although GUS activity appeared to be weaker than with the other three constructs with longer 5' regions (data not shown). This was consistent with the earlier observations that induction of the PR-2d(-607)/GUS construct by TMV infection or SA treatment was two- to threefold lower than that of the longer promoter constructs.

Expression of the PR-2d(-321)/GUS and PR-2d(-171)/GUS genes was not detected in any tissue of the transgenic

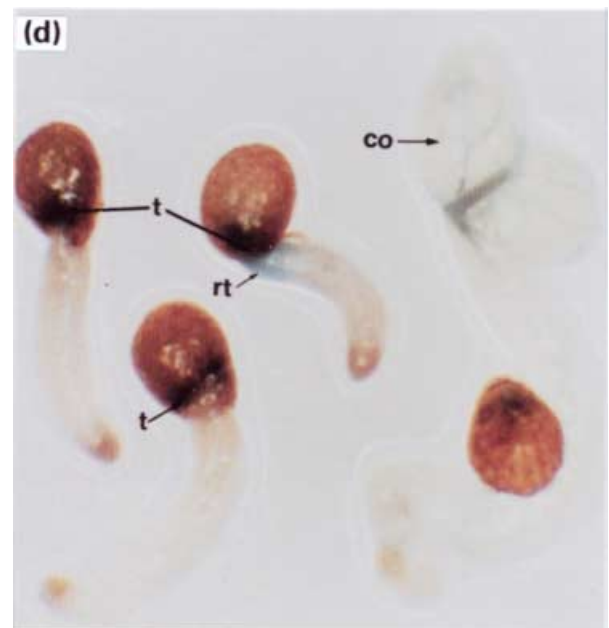
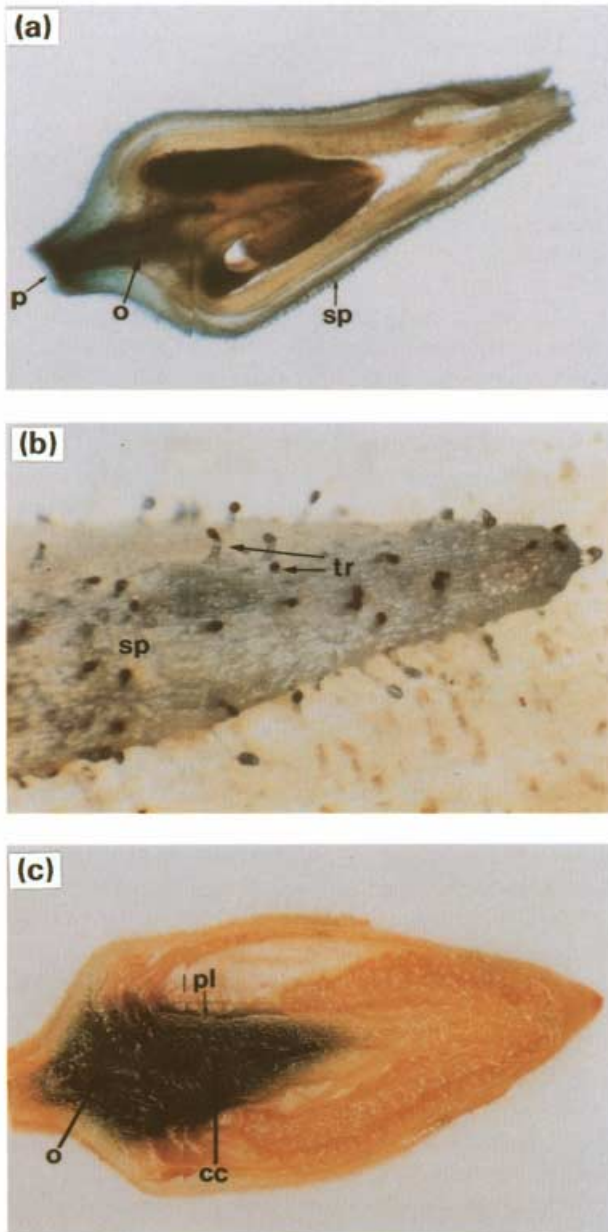


Figure 6. Histochemical localization of GUS activity in floral organs and young seedlings of transgenic PR-2d(-1706)/GUS tobacco.

The general pattern of GUS activity is shown in longitudinal sections through flowers during anthesis (a) or 1 week post-anthesis (c). GUS activity in the upper part of the sepal and in the trichomes during anthesis is shown in (b) while (d) shows the location of PR-2d/GUS expression in young seedlings at 3 days after planting. Indigo dye deposition was observed in the flower sections and seedlings vacuum infiltrated with X-Gluc solution after overnight incubation under conditions described in the legend to Figure 4.

cc, central column; co, cotyledons; o, ovary; p, pedicel; pl, placenta; rt, root; sp, sepal; t, testa; tr, trichomes.

plants. Thus, as for TMV or SA inducibility, a *cis*-acting element(s) necessary for tissue-specific and developmental regulation of PR-2d gene appears to be located between -321 and -607 in its promoter.

Discussion

In this study we describe a set of six chimeric genes constructed by the fusion of various portions of a β -1,3-glucanase (PR-2d) promoter to the GUS reporter gene. The expression of these chimeric genes was studied in transgenic tobacco plants to determine the regions of the promoter required for induction. Using this approach we

have examined expression of this gene after SA treatment or TMV infection. In addition, the tissue-specific and developmental-specific expression of this glucanase gene has been characterized.

Transformants containing a chimeric gene with 607 bp or more of PR-2d 5' flanking sequence showed substantial increases in GUS activity after TMV inoculation. This induction initially occurred in the tissues immediately surrounding the necrotic lesions (Figure 4a) and is similar to results from earlier studies on TMV-induced expression of the PR-1 genes (Antoniw and White, 1986; Ohshima *et al.*, 1990). Later in infection, expression of the PR-2d gene extended into tissues more distant from the lesions (Figure

4b) and eventually into uninoculated leaves (Table 1). This concurs with the reported accumulation of endogenous PR-2 proteins in both inoculated and uninoculated leaves (Côté *et al.*, 1991; Ward *et al.*, 1991b).

Interestingly, during the late stages of the lesion development, GUS activity spread not only to a larger area but could be readily observed in the vasculature near the infection sites (Figure 4b). It is known that β -glucan is deposited in the phloem during physical stress and during pathogen attack, perhaps to prevent the spread of phloem mobile pathogens (Goodman *et al.*, 1986). It is possible that the vascular localization of β -glucanase activity is associated with this phenomenon.

We found that the PR-2d promoter was strongly induced by treatment of the leaves with SA. To define more precisely the region of the promoter required for induction by SA and by TMV infection, a series of 5' deletions was made in the upstream region of the PR-2d promoter. At least two regions are involved. First, SA and TMV induction of the PR-2d(-607)/GUS plants was approximately twofold lower than in the PR-2d(-1706)/GUS, PR-2d(-1462)/GUS and PR-2d(-1047)/GUS plants, suggesting that sequences upstream of the position -607 are required for full induction of the PR-2d gene. Second, in transgenic plants with the PR-2d(-321)/GUS construct the chimeric gene was expressed at only very low levels (1.5- to 3-fold) and showed little, if any, SA inducibility compared with the untransformed controls. From this result we conclude that the -607 to -321 region of PR-2d promoter contains the major element(s) necessary for SA-inducible expression. A similar, though less comprehensive analysis of the PR-2b promoter by Van de Rhee (1992) also demonstrated that a region between -650 and -300 (from the translational start site) was required for moderate- to high-level inducibility by SA. The position of the putative SA responsive region (SARR) in PR-2d is similar to that of the 46 bp (-689 to -643) sequence defined as crucial for SA inducibility of the similarly regulated PR-1a promoter (Van de Rhee *et al.*, 1990). Recent studies (Van de Rhee and Bol, 1993) indicated that at least four regions of the PR-1a promoter, located between -902 and +29 of the start of transcription, are important for SA-regulated expression of this gene. A more detailed analysis will be needed to determine whether the -607 to -321 region of the PR-2d gene also contains more than one element involved in regulating glucanase expression. While, direct sequence comparison did not reveal any obvious sequence similarities between these two promoters, very short regions of homology that are not completely conserved may have gone undetected.

Our analysis of the PR-2d promoter and similar analyses of the PR-1a promoter (Van De Rhee and Bol, 1993; Van De Rhee *et al.*, 1990) indicate that the SARR and TMV response elements of these promoters are identical or share overlapping sequences. This supports the hypothesis

that SA is part of the signal transduction pathway leading from TMV infection to defense-related gene expression (Malamy *et al.*, 1990) since this model predicts that the same promoter element should respond to both pathogen attack and exogenous SA treatment.

It is not yet clear if PR gene expression in flowers, including acidic β -glucanase expression, is involved in plant defense or if this gene has some other physiological function. In this study we confirm the previously reported localization of acidic glucanase activity in the sepal (Côté *et al.*, 1991). At present, four of the five families of PR proteins (PR-1, PR-2, PR-3 and PR-5) have been detected in the sepal tissue of tobacco (Côté *et al.*, 1991; Kononowicz *et al.*, 1992; Lotan *et al.*, 1989; Trudel *et al.*, 1989). Hence, the coordinated regulation of the PR genes observed after TMV infection of leaves is also seen in healthy sepal tissue. However, it should be noted that in other floral organs different patterns of expression of the various PR genes were observed (Cutt *et al.*, unpublished results; Lotan *et al.*, 1989).

Before and during anthesis of the developing flower, high levels of GUS activity were also found in the pedicel and ovary of the flower and in the trichomes of sepals (Figure 6). Expression of the basic PR-5 (osmotin) gene has also been reported in the trichomes of sepals in transgenic PR-5/GUS tobacco plants (Kononowicz *et al.*, 1992). We did not observe GUS activity in the trichomes of the leaves, even during the hypersensitive response following TMV inoculation. The function of the glucanase in the sepal trichomes is unknown; perhaps it plays a protective role.

The tissue specificity of the PR-2d-directed GUS activity changed during the course of floral development. At 1 week post-anthesis GUS activity was still detectable in the sepal, but the majority of the activity was localized to the central part of the flower corresponding to the central column and placenta (Figure 6). In our early study (Côté *et al.*, 1991) we did not detect PR-2 mRNA in the placenta. There are two likely explanations for this discrepancy. In the present study GUS activity was very high in the central column which is adjacent to the placenta. Perhaps slight diffusion of the enzyme or its product into neighboring tissue accounts for the apparent PR-2d/GUS expression in the placenta. Alternatively, the endogenous PR-2 genes may contain a sequence (silencer) which suppresses expression in the placenta. If this presumptive silencer were located greater than 1706bp 5' of the transcriptional start site of the PR-2d gene, its absence in the PR-2d (-1706)/GUS construct could explain the ectopic expression of this chimeric transgene.

Although no GUS activity was observed during seed development, a very specific pattern of GUS activity was exhibited 3-4 days post-germination in the seed coat and in the vascular system of cotyledons (Figure 6). Glucanase

activity in germinating barley seeds has also been reported (Stuart *et al.*, 1986). Perhaps the glucanase encoded by the PR-2d gene is involved in hydrolysis of the seed coat during germination. It is also possible that this enzyme can play an important role in protecting the growing embryo against pathogen attack.

Although proteins with β -glucanase activity have been reported in the stigma and style (Ori *et al.*, 1990), we were unable to detect PR-2d(-1706)/GUS activity in these organs. This is consistent with our previous primer extension analysis which indicated that the glucanase gene(s) expressed in the stigma/style was distinct from the acidic PR-2c gene (Côté *et al.*, 1991). This is also in accordance with the conclusion of Ori and coworkers (1990) that the major, if not only, expressed glucanase genes in the style encode the stylar-specific SP41 glycoproteins. PR-2d/GUS activity was not detected in other parts of the flower or plant.

Organ- and tissue-specific expression was also analyzed in transgenic plants containing the various 5' promoter deletion PR-2d/GUS constructs. The same patterns of expression were seen for constructs which contained 607 bp or more of the 5' flanking region. In contrast, tissue-specific GUS activity could not be detected in plants containing PR-2d/GUS genes with shorter promoter regions (-321 or -171). Thus, the same region required for SA and TMV inducibility appears to be involved in tissue-specific expression. This suggests that there may be some relationship between the signal transduction pathways by which the glucanase gene is induced in each of these situations. There is already considerable evidence that SA is involved in the induction of the PR genes following TMV infection. However, the relationship of SA to expression of PR-2 genes during flowering is unclear. While the early work of Cleland and others indicated that exogenous SA induced flowering in some plant species, SA was not active in other species and no change in its endogenous levels could be detected during the transition from vegetative growth to flowering (Cleland, 1974; for review see Malamy and Kleissig, 1992). Raskin *et al.* (1987) provided the first conclusive evidence that SA regulates certain events during floral development (e.g. thermogenesis in voodoo lilies). However, whether or not SA is involved in the induction of PR genes in floral organs has yet to be determined. The differential regulation of the PR-2 and PR-1 genes in various floral organs during development (Cutt *et al.*, 1991) make this scenario difficult to reconcile. Alternatively, since the control region defined in this study is rather large (286 bp), it could contain several regulatory elements which might play different, and perhaps independent, functions in developmental versus pathogen- (or SA-) induced expression. Further work will be needed to distinguish between these alternative explanations.

Experimental procedures

Plant material and growth conditions

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

Isolation of the PR-2d promoter

Total genomic DNA isolated from *N. tabacum* cv. Xanthi nc leaf tissue was digested with *EcoRI* and fractionated by electrophoresis on a 1.0% agarose gel (Figure 1). DNA ranging in size from 2.2 to 2.9 kb was excised and purified using Gene Clean (BIO101, CA). 0.1 μ g of DNA, diluted to final concentration of 0.5 μ g ml⁻¹, was incubated with T4 DNA ligase overnight at 14°C (Sambrook *et al.*, 1989). Self-ligated circular DNA was subjected to 31 cycles of PCR using the following temperature profile: 94°C, 1.25 min; 48°C, 1.0 min; 70°C, 4.5 min. The two oligonucleotides used in the PCR reaction were primer 1 (5' **GGAATTC**CAGCATTGAAGA CATTGTTTCTGGATAG 3') containing 28 nt corresponding to the sense strand of the PR-2c gene cDNA and primer 2 (5' **GGAATTC**CCATTGAGATCATTCTCGATGTCCCAAAT 3') containing 28 nt complementary to the sense strand; a sequence containing the *EcoRI* restriction site (bold) was included on the 5' termini of both primers to facilitate cloning. Amplification products were digested with *EcoRI* and separated on a 1.0% agarose gel. The major 2.2 kb band was isolated and cloned into the *EcoRI* site of pUC119 (Viera and Messing, 1987). The resulting plasmid was designated pGluc-2. The DNA sequence of the 2.2 kb cloned fragment (Figure 2) was determined using the dideoxy method following the Sequenase version 2 protocol (USB, OH).

Construction of the PR-2d/GUS fusion genes

The pGluc-2 construct was modified by creating a *NcoI* site at the ATG translation initiation codon using the Kunkel method (Sambrook *et al.*, 1989). A deletion series of the 5' flanking region of PR-2d gene was made by exonuclease III treatment (Sambrook *et al.*, 1989) of the pGluc-2 vector after digestion with *PstI* and *BamHI*. The position of each deletion end point was determined by DNA sequencing. The full-length construct and deletions corresponding to positions -1462, -1047, -607, -321 and -171 from the PR-2d transcriptional initiation site were then fused with GUS/3'NOS sequences to form chimeric constructs. The *NcoI-SstI* fragment from the pRAJ 275 vector (Jefferson *et al.*, 1986) containing GUS coding sequence was ligated with 3' nopaline synthase (NOS) sequence from pBI101.2 (Clontech Laboratories Inc., CA). Then the GUS/3'NOS construct was inserted into the *NcoI-EcoRI* sites of the pGluc-2 or deletion derivatives of it.

Tobacco transformation

The expression cassettes containing the PR-2d(-1706)/GUS gene fusion and the respective deletion derivatives were cloned as *EcoRI-HindIII* fragments into the binary vector pGA482 (An, 1986) and mobilized into *Agrobacterium tumefaciens* strain LBA4044 by triparental mating (Ditta *et al.*, 1980). Leaf discs from *N. tabacum* cv. Xanthi nc plants were transformed and plants were regenerated by standard methods (Horsch *et al.*, 1985). Control plants were constructed by transformation with the chimeric CaMV 35S promoter/GUS gene/3'NOS gene from pBI121 vector (Clontech Laboratories Inc., CA). Primary transformants were

allowed to self-fertilize and F₁ seeds were collected and germinated on Murashige and Skoog agar medium (GIBCO BRL, MD) with 300 µg ml⁻¹ kanamycin sulphate. Transgenic seedlings were transferred to soil.

Induction of the PR-2d/GUS genes

Six- to eight-week-old plants were used for GUS assays and RNA extraction following treatment with SA or inoculation with TMV. For induction by SA, 1 cm discs were punched out from leaves of transgenic plants and floated in a Petri dish on H₂O or on 1.0 mM SA, pH 6.7, for 48 h at 22°C (14 h light, 8 h dark cycle). After incubation, leaf discs were frozen and stored at -70°C. For inoculation by TMV, leaves of plants were dusted with carborundum and rubbed with a 1 µg ml⁻¹ suspension of TMV strain U1 in 50 mM phosphate buffer, pH 7.5. Leaf discs were taken from inoculated leaves at 0, 2 and 4 days after inoculation and upper uninoculated leaves at 2, 4, 6 and 8 days after inoculation.

Detection of GUS activity

β-glucuronidase activity was determined using either a fluorometric assay or by histochemical staining. For the fluorometric GUS assay, leaf discs were homogenized in 0.5 ml lysis buffer. The homogenate was centrifuged for 10 min in an Eppendorf centrifuge at 5°C, and GUS activity of the supernatant was measured, essentially as described by Jefferson *et al.* (1987). GUS activity was normalized to protein concentration for each crude extract and calculated as pmol of 4-methyl umbelliferone (4-MU) produced per minute per milligram of soluble protein. Protein content was measured by the Bradford (1976) method using BSA as a standard.

For histochemical staining hand-cut tissue sections were vacuum infiltrated with a staining solution containing 1 mg ml⁻¹ X-Gluc (5-bromo-4-chloro-3-indolyl β-glucuronide), 100 mM phosphate buffer, pH 7.0, 5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆], 10 mM EDTA and incubated for 4–20 h at 37°C. After staining, the tissue sections were fixed by incubation in a solution of 5% formaldehyde, 5% acetic acid, 20% ethanol for 10 min at room temperature and then cleared by incubation sequentially in 50% ethanol for 30 min and 100% ethanol overnight at room temperature. Stained sections were viewed and photographed with the Wild M650 Stereomicroscope.

Primer extension analysis

Total RNA was prepared as described by Berry *et al.* (1985) from untreated and SA-treated untransformed Xanthi nc or PR-2d(-1706)/GUS transgenic plants. The primer extension protocol described by Dunsmuir *et al.* (1987) was used to determine the transcriptional start site for the PR-2d (and PR-2c) gene. Primer 1 (which was used for IPCR and is complementary to the PR-2c mRNA) or the 25-mer oligonucleotide 5'-TCCAGACTGAA TGCCACAGGCCGT-3' (which is complementary to the sense strand of the GUS gene (Jefferson *et al.*, 1986)) were annealed to total RNA at 45°C for the extension reactions.

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