The Plant Cell, Vol. 2, 1131–1143, December 1990

Tissue-Specific and Pathogen-Induced Regulation of a *Nicotiana plumbaginifolia* β-1,3-Glucanase Gene

Carmen Castresana, Fernanda de Carvalho, Godelieve Gheysen, Marianne Habets, Dirk Inzé, and Marc Van Montagu[1]

Laboratorium voor Genetica, Rijksuniversiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

The *Nicotiana plumbaginifolia gn1* gene encoding a β-1,3-glucanase isoform has been characterized. The *gn1* product represents an isoform distinct from the previously identified tobacco β-1,3-glucanases. By expressing *gn1* in *Escherichia coli*, we have determined directly that the encoded protein does, indeed, correspond to a β-1,3-glucanase. In *N. plumbaginifolia*, *gn1* was found to be expressed in roots and older leaves. Transgenic tobacco plants containing the 5′-noncoding region of *gn1* fused to the β-glucuronidase (GUS) reporter gene also showed maximum levels of GUS activity in roots and older leaves. No detectable activity was present in the upper part of the transgenic plants with the exception of stem cells at the bases of emerging shoots. The expression conferred by the *gn1* promoter was differentially induced in response to specific plant stress treatments. Studies of three plant-bacteria interactions showed high levels of GUS activity when infection resulted in a hypersensitive reaction. Increased gene expression was confined to cells surrounding the necrotic lesions. The observed expression pattern suggests that the characterized β-1,3-glucanase plays a role both in plant development and in the defense response against pathogen infection.

INTRODUCTION

A group of proteins designated pathogenesis-related (PR) proteins have been shown to be synthesized by plants in response to microbial infection (Collinge and Slusarenko, 1987; Evered and Harnett, 1987). The induction of PR proteins is commonly associated with the establishment of an incompatible interaction manifested by the appearance of a hypersensitive reaction in the plant (Fritig et al., 1987). However, definitive evidence of a defined contribution of PR proteins to the plant defense mechanisms has not been obtained.

PR proteins were initially characterized in virus-infected tobacco plants as a set of low-molecular-weight proteins, selectively extracted at low pH, resistant to proteases, and localized in the intercellular spaces of leaves (van Loon and van Kammen, 1970; Antoniw et al., 1980; van Loon, 1985). Additional information has significantly modified the original concept of PR proteins. Furthermore, PR proteins recently have been identified in multiple plant species, including both monocotyledonous and dicotyledonous plants (Carr and Klessig, 1990). On the basis of their serological relationships and more recently on their partial amino acid sequences, PR proteins are now grouped into five independent families of closely related proteins (van Loon et al., 1987). Each family comprises both acidic as well as basic isoforms (Rigden and Coutts, 1988; Carr and Klessig, 1990). Furthermore, whereas some of these proteins are indeed extracellular, other members of the same family have been shown to be confined to the vacuoles (Boller and Vögeli, 1984; Van den Bulcke et al., 1989). Protein sequence analysis and cloning of the corresponding cDNAs have revealed that the proteins detected are products of different structural genes, and not the result of different post-translational modifications (Bol et al., 1987).

Several PR proteins have been identified as β-1,3-glucanases (Kauffmann et al., 1987; Kombrink et al., 1988; Joosten and De Wit, 1989). A role for these hydrolytic enzymes as part of the plant defense response has been suggested on the basis of their inhibitory effect in combination with chitinases on the in vitro growth and/or sporulation of pathogenic fungi (Boller, 1987; Mauch et al., 1988a). Similarly, it has been postulated that β-1,3-glucanases could contribute actively to the induction of the defense response by releasing elicitors from cell walls of pathogenic microorganisms (Keen and Yoshikawa, 1983). However, a possible role in more fundamental aspects of plant cell metabolism has not been excluded.

The partial amino acid sequences corresponding to five distinct tobacco β-1,3-glucanase isoforms, both acidic (extracellular) and basic (intracellular), have been reported (Van den Bulcke et al., 1989), and a different pattern of expression for both groups of isoforms has been described

[1] To whom correspondence should be addressed.

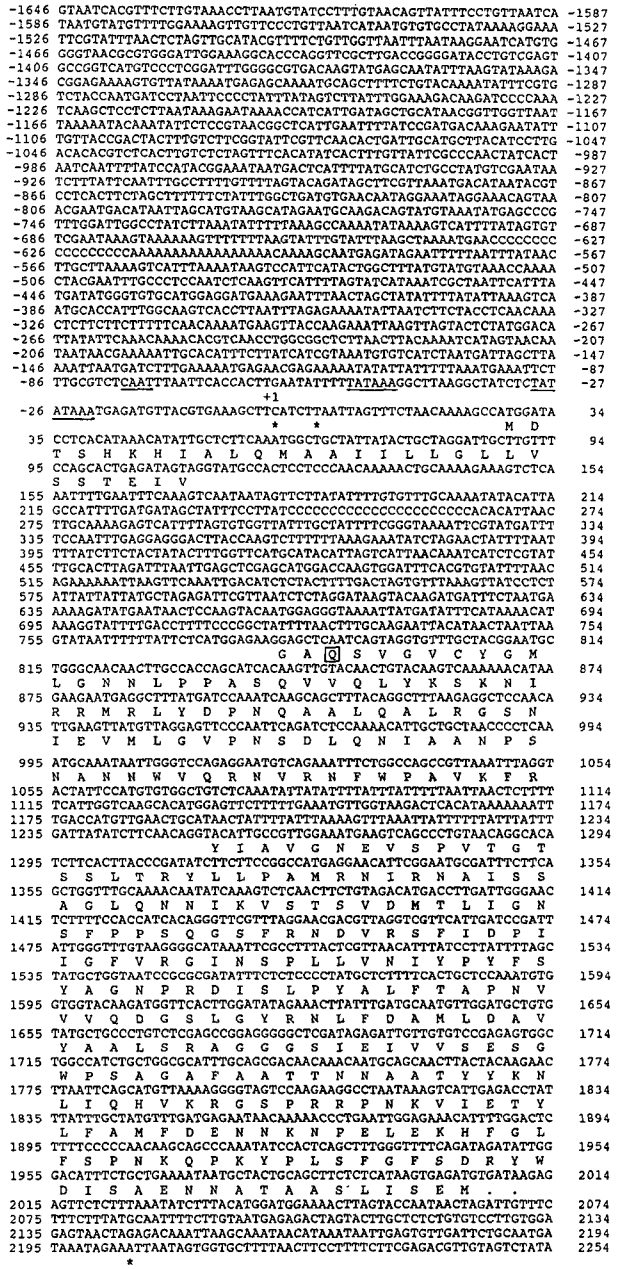


Figure 1. Nucleotide and Deduced Amino Acid Sequence of the *N. plumbaginifolia gn1* Gene (GenBank Accession No. M38281).

Nucleotides are numbered with the cap site designated +1; putative TATA and CAAT boxes are underlined. The glutamine corresponding to the first amino acid of the mature GN1 protein is boxed (□). Transcription start points (+1 and +6) and the polyadenylation site (+2204) are indicated with an asterisk (*).

(Memelink et al., 1990). β-1,3-Glucanases have been shown to be induced not only after pathogen infection, but also as a result of different chemical and hormonal treatments (Mohnen et al., 1985; Felix and Meins, 1987; Mauch et al., 1988b; Vögeli et al., 1988; Vögeli-Lange et al., 1988). In addition, β-1,3-glucanases have been shown to be present in both root and floral tissues of healthy tobacco plants (Felix and Meins, 1986; Lotan et al., 1989).

In spite of the extensive information accumulated, the contribution and possible function of the specific isoforms, both in healthy plants and in response to plant stress, remain uncertain. Similarly, it is not known whether the expression of specific isoforms is mediated through activation of a common or partially independent signal transduction pathway.

To understand better the regulatory mechanisms controlling the expression of plant defense-related genes, the β-1,3-glucanase *gn1* gene isolated from *N. plumbaginifolia* plants has been examined in detail. Expression of the endogenous gene in *N. plumbaginifolia* and a reporter β-glucuronidase gene under the control of the *gn1* promoter in transgenic tobacco plants has been examined. Expression has been determined both in healthy plants and in plants responding to various stress treatments. In addition, a detailed histological analysis has been performed to identify the specific cells in which expression mediated by the *gn1* promoter takes place.

RESULTS

Molecular Characterization of the *N. plumbaginifolia gn1* Gene

A genomic library of *N. plumbaginifolia* was screened using a β-1,3-glucanase cDNA as a probe (De Loose et al., 1988). A positive phage containing a 10.2-kb DNA insert was isolated and characterized by hybridization, restriction, and sequence analysis (De Loose et al., 1988). From these studies, a putative β-1,3-glucanase gene, *gn1*, present in a 3.5-kb HindIII DNA fragment, was identified and analyzed in detail to determine its molecular, functional, and expression characteristics. The complete nucleotide sequence of *gn1*, including sequences corresponding to the 5′-flanking and 3′-flanking regions of the gene, are shown in Figure 1. *gn1* is identical to the utilized cDNA. The coding region of *gn1* is contained in three exons and yields a precursor protein of 370 amino acids. The encoded protein contains a 30-amino-acid N-terminal extension presumably involved in its targeting to the endoplasmic reticulum (De Loose et al., 1988). Two intervening sequences

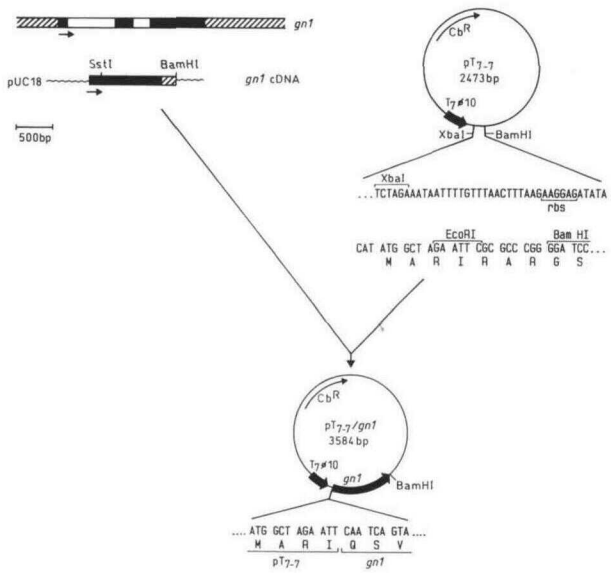


Figure 2. Construction of the pT7-7/*gn1* Fusion.

The vector pT7-7 containing the strong T7 RNA polymerase promoter was restricted with EcoRI, blunt ended with the Klenow fragment of DNA polymerase I, and then restricted with BamHI. The plasmid pUC18 containing the cDNA derived from the *gn1* gene (De Loose et al., 1988) was linearized at the SstI restriction site, blunt ended with T4 DNA polymerase, and then restricted with BamHI. The 1120-bp resulting fragment containing the DNA sequences corresponding to the mature protein and the 3′-untranslated region of *gn1* was purified from an agarose gel and ligated to the restricted pT7-7 vector. Correct insertion in pT7-7 was determined by sequencing. The resulting recombinant plasmid pT7-7/*gn1* was introduced in *E. coli* cells where the activity of the encoded protein was determined. Cloning at the pT7-7 EcoRI/BamHI sites produced a translational fusion containing the first 4 amino acids of the T7 gene 10 protein fused to the mature GN1 protein. The *gn1* sequences are represented as follows: ▨, noncoding flanking sequences; ■, coding region. The arrows indicate the direction of gene transcription.

extending for 669 nucleotides and 199 nucleotides are present after codons 28 and 119, respectively.

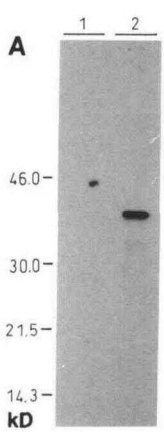
The deduced amino acid sequence of the protein encoded by *gn1* has been compared with the corresponding partial amino acid sequences of different intracellular and extracellular β-1,3-glucanase isoforms, previously characterized in tobacco plants (Shinshi et al., 1988; Van den Bulcke et al., 1989). The *gn1* gene product shows 60% to 65% sequence identity to the three extracellular β-1,3-glucanase isoforms characterized, and 70% identity to the two vacuolar isoforms identified in tobacco plants. These results revealed that the protein encoded by the *N. plumbaginifolia gn1* gene probably does not correspond to the previously sequenced proteins, but in contrast represents a different glucanase isoform. This argument is supported by sequence analysis of a second β-1,3-glucanase gene (*gn2*) isolated from *N. plumbaginifolia* that shares 95% identity with the intracellular isoforms of tobacco and that more likely represents the corresponding gene of the tobacco intracellular isoforms in *N. plumbaginifolia* (Gheysen et al., 1990).

The Protein Encoded by *gn1* Is a Hydrolytic Enzyme with β-1,3-Glucanase Activity

To determine directly whether the *gn1* gene product corresponded to a β-1,3-glucanase enzyme, the *gn1* cDNA lacking its signal sequences was expressed in *Escherichia coli* under the control of the T7 promoter. Figure 2 is a diagram of the recombinant plasmid pT7-7/*gn1* constructed to analyze the enzymatic activity conferred by the GN1 protein.

As shown in Figure 3A, induction of *gn1* expression in the presence of ^{35}S-methionine resulted in the production of a unique approximately 37-kD protein in the bacterial cells carrying the pT7-7/*gn1* plasmid, but not in the control cells. As estimated by Coomassie Blue staining, the protein encoded by *gn1* represented approximately 0.5% to 1% of the total cell protein. In addition, the identified protein cross-reacted strongly with antiserum raised against a β-1,3-glucanase enzyme purified from tobacco plants (data not shown).

The enzymatic activity of the protein produced in *E. coli* was determined by detection of the reducing sugars re-



B

β(1, 3)-glucanase activity in *E.coli*

mmoles glucose mg^{-1} protein hour^{-1}	
control	0.28
gn1	3.65

Figure 3. Analysis of the T7-7/*gn1* Encoded Protein in *E. coli* Cells.

(A) SDS-PAGE analysis of *E. coli* lysates after induction of the T7 polymerase promoter expression in the presence of ^{35}S-methionine. Lane 1, cells containing the pT7-7 vector; lane 2, cells containing the pT7-7/*gn1* plasmid. Positions of molecular weight markers are indicated (in kilodaltons).

(B) β-1,3-Glucanase activity in the supernatant fraction of bacterial cell lysates. The activity detected in *E. coli* cells containing the construct pT7-7/*gn1* was compared with the activity corresponding to *E. coli* cells containing the vector pT7-7 utilized as a control.

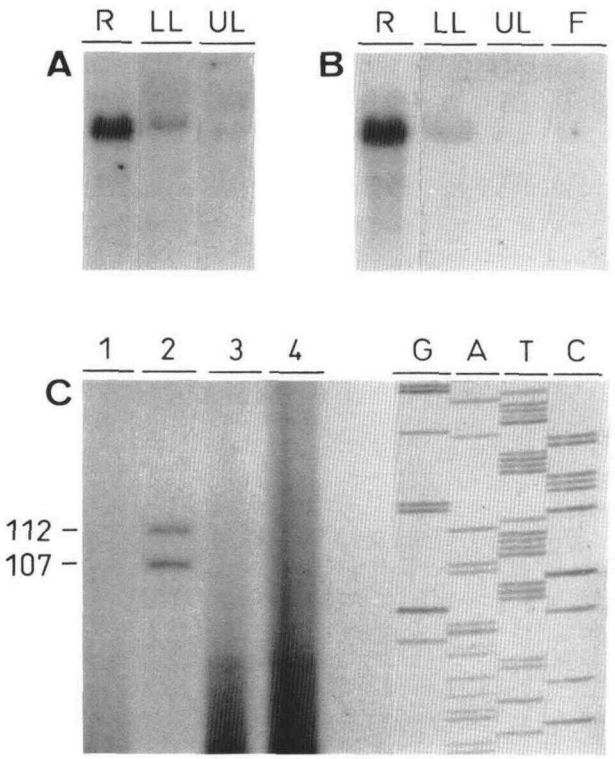


Figure 4. Analysis of *gn1* Expression on *N. plumbaginifolia*.

(A) RNA gel blot analysis of total RNA from roots (R), lower leaves (LL), and upper leaves (UL) of preflowering plants.

(B) RNA gel blot analysis of total RNA from roots (R), lower leaves (LL), upper leaves (UL), and flowers (F) of plants with fully developed flowers.

(C) Primer extension analysis was carried out using a *gn1*-specific oligonucleotide. The 5′-end-labeled primer was hybridized overnight at 30°C to 50 μg of roots (lane 2), upper leaves (lane 3), and *E. coli* RNA (lane 4). No RNA was included in the hybridization sample corresponding to lane 1. The size of the extended products was estimated by reference to the mobility of a known DNA sequence (lanes G, A, T, and C).

leased by the enzyme after incubation with the substrate laminarin. The results revealed a strong β-1,3-glucanase activity in the supernatant fraction obtained after sonication of the *E. coli* expressing the GN1 protein. In contrast, only a weak activity was detected in the control cells containing the plasmid pT7-7 (Figure 3B).

Expression of the β-1,3-Glucanase *gn1* Gene and Mapping of the Corresponding Transcript in *N. plumbaginifolia* Plants

β-1,3-Glucanase enzymes have been shown to be induced in several plant species in response to multiple treatments. To evaluate the expression characteristics of *gn1*, we assessed the abundance of the corresponding mRNA in *N. plumbaginifolia* by high-stringency RNA gel blot analyses. These investigations showed that *gn1* hybridized almost exclusively to transcripts present in the root tissue of healthy, untreated plants. As shown in Figure 4A, a low hybridization signal was also detected in the RNA from the older leaves of the plant.

Although some PR proteins have been reported to be induced during plant flowering (Fraser, 1981; Lotan et al., 1989), *gn1* mRNA levels did not increase in leaf and root tissues of plants with fully developed flowers. Moreover, no RNA was detected in the floral organs of the plant (Figure 4B).

Primer extension analyses were carried out to define the cap site for the transcript derived from *gn1*. A synthetic oligonucleotide complementary to nucleotides 45 to 85 downstream from the first ATG of the coding sequence was 5′-end labeled and hybridized to RNA from upper leaves and root tissues of preflowering plants. After extension with reverse transcriptase, two DNA fragments of 107 nucleotides and 112 nucleotides were obtained when the oligonucleotide was hybridized to RNA isolated from root tissue; no signal was detected for leaf mRNA (Figure 4C). These analyses confirm the expression pattern characterized for the gene *gn1* by RNA gel blot analyses. Although at this stage we cannot exclude the possibility that the probe utilized hybridized to very closely related transcripts, further experiments clearly demonstrated the specificity of the utilized oligonucleotide. The results obtained define two putative cap sites at 22 bp and 27 bp upstream from the first ATG of the coding region. The significance of these two sites is not known. In Figure 1, we have designated the more upstream of the two sites as position +1. The sequence TATAAA, likely to correspond to the *gn1* TATA box, resides at position −22 relative to this site.

Construction of a *gn1* Promoter-β-Glucuronidase Fusion and Analysis of the Expression Conferred in Transgenic Tobacco Plants

To facilitate the study of the regulatory characteristics conferred by the *gn1* promoter, a chimeric construct was prepared by fusing 2 kb of 5′-flanking sequence of *gn1* to the coding region of the bacterial β-glucuronidase (GUS) gene, as shown in Figure 5. This construct was introduced into tobacco cells and after regeneration, randomly selected transgenic plants were assayed for GUS activity.

The results obtained by analysis of 10 independent transgenic plants are shown in Figure 6. The 5′-flanking sequence of the *gn1* gene conferred high levels of GUS activity in the roots of the transformed plants. In accordance with the results obtained for the endogenous

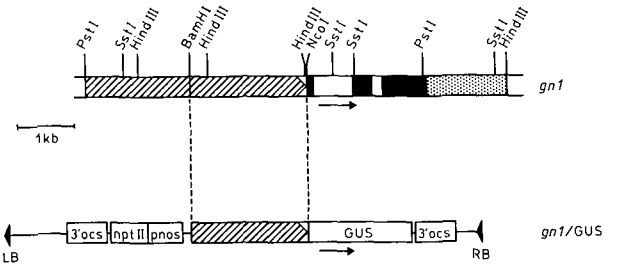


Figure 5. Construction of the *gn1*/GUS Fusion.

A BamHI/NcoI DNA fragment extending from the initiating ATG of *gn1* to −2000 bp in the 5′-flanking region was fused to the coding region of the *gus* reporter gene (Jefferson et al., 1987). The chimeric construct was inserted in the BamHI site of pDE1001, and the resulting recombinant plasmid was transferred to *A. tumefaciens* and introduced into tobacco cells by leaf disc transformation. The *gn1* sequences are represented as follows: ▨, 5′-flanking region; ■, coding region; and ▩, 3′-flanking region. LB and RB represent the left and right borders of the T-DNA, respectively. The arrows indicate the direction of transcription of the *gn1* and *gus* genes driven by the *gn1* promoter.

N. plumbaginifolia gene, the *gn1* promoter directed the expression of the GUS reporter gene in the lower leaves of the transgenic plants. In addition, smaller but significant levels of GUS activity were detected in the adjacent leaves of the plant. A gradient of activity was established through the plant with maximum values corresponding to the roots and older leaves, whereas no detectable activity was present in the upper leaves of the plant. A maximum variation of up to 10-fold was determined for the absolute levels of GUS activity among the independent transformants examined. However, the ratio of GUS activity between the different regions of the plant analyzed remained constant in all individual plants.

To identify the specific cells expressing the *gn1*/GUS construct, the spatial distribution of GUS activity was determined histochemically by an in situ assay. Two representative transgenic plants with average values of GUS activity were selected for these experiments. As shown in Figure 7, dark-field micrographs revealed in purple light the presence of GUS enzyme activity in the cells of the plant tissues examined. Extensive staining was detected in roots and in the lower stem and leaves (Figures 7A, 7B, and 7C). In roots, the *gn1*/GUS construct was highly expressed in the parenchymatic cells and in the cells of the phloem vessels. In contrast, no staining was observed in the epidermis and xylem (Figure 7C). A homogeneously distributed staining was observed in the stem and leaves of the lower part of the plant. In both organs, all cells appear to express the gene fusion with the exception only of the xylem vessels, where no staining was detected (Figures 7A and 7B). In contrast to the results observed in the roots, where no activity was observed in the epidermis, in both the stem and lower leaves, the GUS gene was strongly expressed in the epidermal cells. This difference could be simply a consequence of the fact that epidermal cells in roots are quite short lived and most are dead (Campbell and Greaves, 1990). Finally, analysis of the upper parts of the plant revealed an almost complete absence of expression. An exception to this was found for the stem cells at the base of the emerging shoots, where significant levels of activity were clearly apparent. The spatial distribution of GUS activity was identical in the tissues of the two transgenic plants examined.

Analysis of the Expression Conferred by the *gn1* Promoter in Response to Plant Stress

To analyze further the regulatory characteristics conferred by the *gn1* promoter, the level of GUS activity was evaluated after subjecting the transgenic plants to different stress treatments known to induce PR proteins.

Salicylic acid treatment has been shown to act as a strong inducer of PR proteins in several plant species (White, 1979; Ohshima et al., 1990; Van de Rhee et al., 1990). Although the physiological role of this chemical compound with respect to induction of PR proteins is unknown, a correlation between levels of salicylic acid and PR protein induction has been demonstrated (Klessig et al., 1989). The effect of this inducer on GUS activity was evaluated before and after spraying the transgenic plants with 5 mM salicylic acid. As shown in Figure 8A, salicylic acid treatment induced expression of this promoter about 14-fold, based upon the increase in levels of GUS activity.

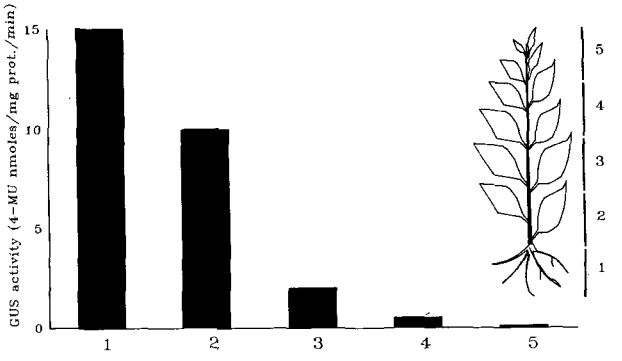


Figure 6. Analysis of GUS Activity in Transgenic Tobacco Plants Containing the *gn1*/GUS Construct.

The data represent the average GUS activity determined for 10 independent transgenic plants examined approximately 12 weeks after transfer to soil. GUS activity was determined in different plant regions designated 1 to 5 as represented in the diagram at the right.

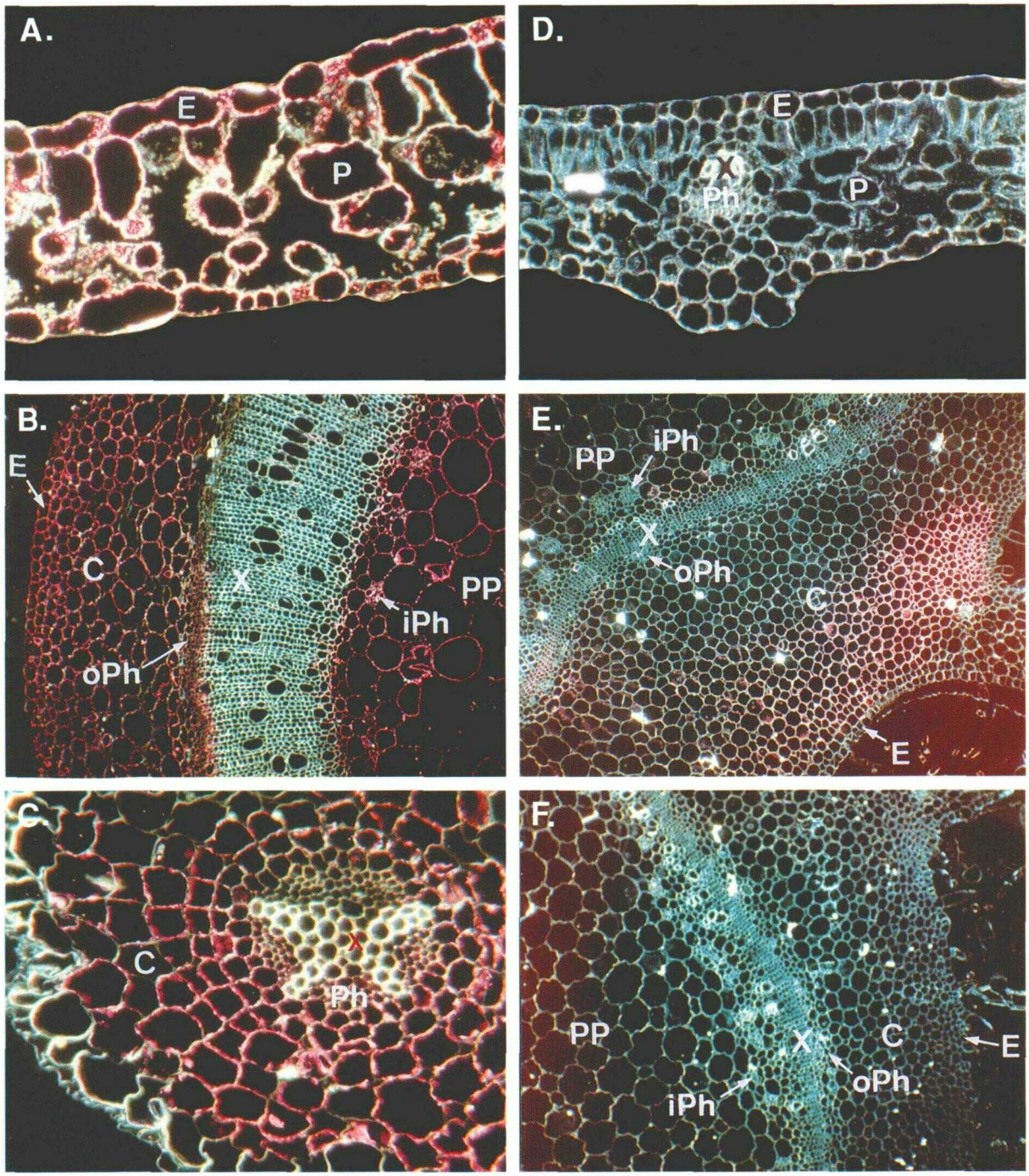


Figure 7. Histochemical Localization of *gus* Gene Expression in Transgenic Tobacco Plants Containing the *gn1*/GUS Construct.

Dark-field micrographs reveal in purple light the presence of GUS enzymatic activity in the cells of the plant tissue examined (approximately 12 weeks after transfer to soil).

(A) Cross-section through the lower leaf of the plant.

(B) Cross-section through the basal part of the stem.

(C) Cross-section through the main root of the plant.

(D) Cross-section through an upper young leaf of the plant.

(E) Cross-section through the upper part of the stem at the base of an emerging secondary shoot.

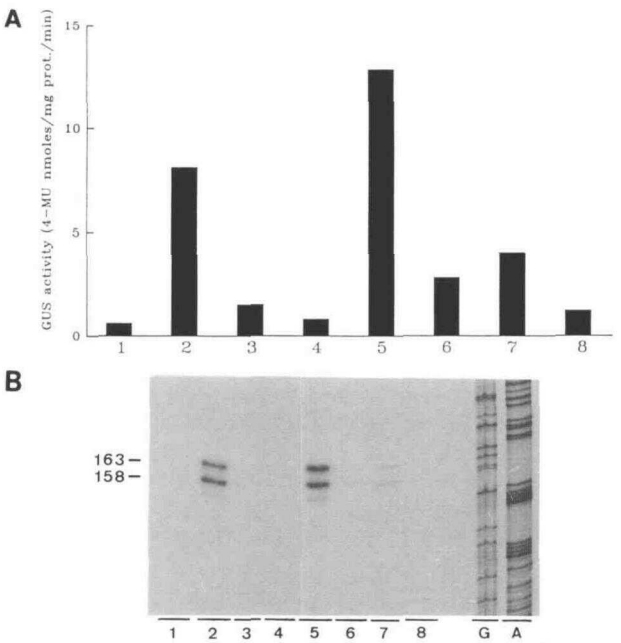


Figure 8. Analysis of Stress-Induced Expression of GUS Activity in Transgenic Tobacco Plants Containing the *gn1*/GUS Construct.

(A) GUS activity was determined in leaf tissue of transgenic plants before and after each of the following stress treatments: 1, nontreated leaves; 2, salicylic acid; 3, ethylene; 4, wounding; 5, *P. syringae* pv *syringae* infection; 6, *E. carotovora* subsp. *carotovora* infection; 7, *P. fluorescens*; and 8, water infiltration.

(B) Primer extension analysis was performed on total RNA prepared from transgenic plants after each of the different stress treatments. An oligonucleotide complementary to nucleotides 106 to 136 of the coding region of the *gus* gene was hybridized to 50 μg of plant RNA. The size of the extended products was estimated by reference to the mobility of a known DNA sequence (lanes G and A).

The role of ethylene as a cellular signal affecting plant response against microbial infection has been postulated on the basis of experimental data showing an increase in ethylene biosynthesis during plant-pathogen interactions (Yang and Hoffman, 1984). It has been shown that plant treatment with ethylene induces the expression of several plant stress-related genes, including genes encoding for PR proteins (Boller et al., 1983; Broglie et al., 1986; Ecker and Davis, 1987). In addition, ethylene treatment produced a strong induction in the expression of a second β-1,3-glucanase gene (*gn2*) from *N. plumbaginifolia* (C. Castresana, unpublished data). The effect of ethylene on the expression directed by the *gn1* promoter was examined. Relative to the strong induction observed with salicylic acid, it was of interest to note that only a weak induction in GUS activity (about 2.5-fold) was detected after continuous flushing with 50 ppm ethylene for 48 hr (Figure 8A).

Wounding has also been shown to induce the expression of several stress-related genes, including tobacco PR proteins (Ohshima et al., 1990). To examine whether mechanical stress had any effect on expression conferred by the *gn1* promoter, GUS activity was determined in fully expanded leaves from transgenic plants after extensive puncturing. A very weak increase (about 1.7-fold) in the level of activity was detected when the plants were analyzed 48 hr after physical damage (Figure 8A). It has been observed that the level of induction may vary significantly during the time period after wounding (Keil et al., 1989; Keller et al., 1989; An et al., 1990; Stanford et al., 1990). Accordingly, independent transgenic plants were used to evaluate the level of GUS activity at shorter periods of time. No significant increase of GUS activity was observed when plants were examined at 5 hr, 10 hr, and 24 hr after mechanical stress (data not shown).

The induction of PR proteins in response to pathogen attack has been shown to be associated mainly with the establishment of an incompatible plant-pathogen interaction. To evaluate a possible role of the GN1 protein in this plant defense reaction, the level of GUS activity in the transgenic plants was examined after inoculation with the incompatible bacterium *Pseudomonas syringae* pv *syringae*. Formation of necrotic lesions characteristic of a hypersensitive reaction was observed in the inoculated area after bacterial infection. GUS analyses showed that the level of expression conferred by the *gn1* promoter was strongly induced during this defense response, as indicated by an approximately 21-fold increase in the level of GUS activity subsequent to infection (Figure 8A).

To examine the specificity of this response, plant leaves were analyzed after inoculation with either the compatible bacteria *Erwinia carotovora* subsp. *carotovora* or a saprophytic strain of *Pseudomonas fluorescens* (Figure 8A). No hypersensitive necrotic lesions were visualized as a consequence of these treatments, and the level of GUS activity determined showed an approximately threefold increase in response to *E. carotovora* subsp. *carotovora* and an approximately 4.5-fold increase for *P. fluorescens*. A low level of induction (about 1.9-fold) was detected after treatment with water (Figure 8A).

Figure 7. (continued).

(F) Cross-section through the internodal region of the upper part of the stem.

The plant tissues observed are indicated as follows: C, cortex parenchyma; E, epidermis; iPh, inner phloem; oPh, outer phloem; P, parenchyma; PP, pith parenchyma; X, xylem.

The *gn1*/GUS Construct Is Regulated at the Level of Gene Activation in Response to Plant Stress

To investigate whether the induction in GUS activity was due to specific expression conferred by the *gn1* promoter and not to induced changes in GUS enzyme stability, primer extension analyses were performed on total RNA prepared from transformed plants after each of the different stress treatments.

An oligonucleotide complementary to nucleotides 106 to 136 downstream from the initiating ATG of the GUS gene was 5′-end labeled and hybridized to total plant RNA. As shown in Figure 8B, two extension products of 158 bp and 163 bp were obtained for RNA from the transgenic plants. The level of these products was in proportion to the level of GUS activity previously evaluated, and, therefore, we conclude that the induction observed was due to gene activation and not to protein stability. Furthermore, the same initiation points for transcription were observed in all cases. The two putative cap sites correspond to those identified for the wild-type *gn1* gene.

In Situ Localization of GUS Activity during the Plant Defense Response

To identify the plant cells expressing the *gn1*/GUS construct during the defense response of the plant, in situ staining for GUS activity was carried out after inoculation of transgenic plants with the incompatible bacteria *P. syringae* pv *syringae*. Bright-field microscopic images revealed that the expression conferred by the *gn1* promoter was locally restricted to the cells surrounding the hypersensitive lesion.

As shown in Figure 9B, GUS activity was not detected inside the inoculated area even when analyzed before the necrotic lesion was externally manifested (6 hr after bacteria inoculation). GUS staining was absent in control leaves from noninoculated transgenic plants (Figure 9A).

DISCUSSION

The *N. plumbaginifolia gn1* gene encoding for a β-1,3-glucanase has been examined at both molecular and functional levels. The structure of the gene has been determined, the enzymatic properties of the encoded protein have been assayed, and the expression characteristics of the gene both in healthy plants and in response to various stress treatments have been characterized.

gn1 Encodes a Novel β-1,3-Glucanase Enzyme

We have previously reported the partial amino acid sequence of both intracellular and extracellular β-1,3-glucanase enzymes isolated from tobacco plants (Van den Bulcke et al., 1989). Comparative sequence analyses revealed that the protein encoded by *gn1* shared 60% to 65% sequence identity with the previously determined extracellular isoforms and 70% identity with the intracellular isoforms identified in tobacco. It seems unlikely that the sequence divergence observed for the protein encoded by the *N. plumbaginifolia gn1* gene and the tobacco β-1,3-glucanase isoforms simply reflects the evolutionary distance between these two plant species. These species are closely related, as shown by the high level of sequence conservation reported for two members of the *rbcS* gene family (Poulsen et al., 1986). In addition, the high conservation determined (95% identity) between a second glucanase gene (*gn2*) from *N. plumbaginifolia* and the intracellular isoforms of tobacco supports this argument (Gheysen et al., 1990). The direct homolog of *gn1* has probably not been characterized in tobacco because of the low level of expression of *gn1* relative to other isoforms (C. Castresana, unpublished data). Accordingly, we conclude that the protein encoded by *gn1* does not correspond to any of the previously characterized β-1,3-glucanases, but represents a new isoform. By expressing *gn1* in *E. coli*, we have determined directly that the encoded protein is a β-1,3-glucanase.

β-1,3-Glucanases Play an Undefined Role in Plant Development

We have characterized the expression pattern of the gene *gn1* in different plant tissues and at different stages of plant development. In *N. plumbaginifolia* plants, *gn1* was expressed in root tissue and at a lower level in the older leaves. Similarly, analyses of transgenic tobacco plants containing the *gus* reporter gene driven by the *gn1* promoter showed a high level of GUS activity in the roots and in the lower stem and leaves of the plant. Lower but significant GUS activity was detected in leaves derived from the middle part of the plant. This activity decreased toward the upper part of the plant and was undetectable in leaves derived from the upper one-quarter of the plant. The expression observed in the leaves from the middle of the transgenic plants was not detected for the endogenous *gn1* in *N. plumbaginifolia*, possibly reflecting the increased sensitivity of the fluorimetric assay and/or the high stability of the GUS protein. A similar expression pattern has been observed in tobacco plants for an intracellular β-1,3-glucanase (Felix and Meins, 1986), and Shinshi et al. (1987) have reported that an intracellular isoform corresponding to the PR chitinase gene family shows analogous regulatory characteristics. In addition, it has been reported that the *Agrobacterium tumefaciens* nopaline and mannopine synthase promoters are also highly active in the lower parts of the plant of transgenic tobacco plants and de-

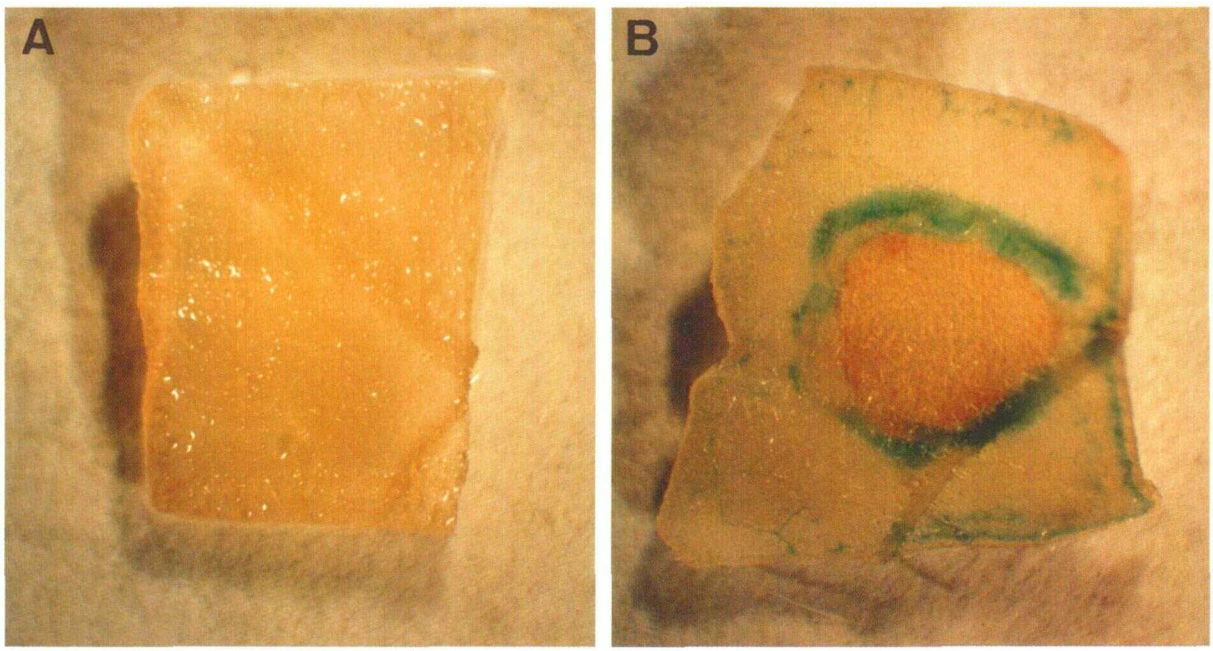


Figure 9. Histological Localization of GUS Activity during the Plant Defense Response.

Bright-field micrographs reveal in blue the presence of GUS enzymatic activity in the cells of the plant tissues analyzed. Transgenic plants containing the *gn1*/GUS construct were examined approximately 12 weeks after transfer to soil. After staining with *X*-glucuronide, the plant sections examined were placed in ethanol to stop the reaction and to remove chlorophyll.

(A) Section of a fully expanded, upper young leaf from a noninfected transgenic plant.

(B) Section of a fully expanded, upper young leaf from a transgenic plant 6 hr after inoculation with the incompatible bacteria *P. syringae* pv. *syringae*.

crease in the upper parts (An et al., 1988; Langridge et al., 1989).

The observation that the appearance of β-1,3-glucanase in tobacco tissues is modified in response to the presence of auxins and cytokinins (Mohnen et al., 1985; Memelink et al., 1987; De Loose et al., 1988) led to the suggestion that the expression pattern observed for those β-1,3-glucanases could be due to a change in the hormonal level through the plant. Although definitive evidence in favor of this suggestion has not been reported, auxins and cytokinins play a central role in regulating plant growth and development (Moore, 1989). It is possible that the presence of β-1,3-glucanases in healthy, unstressed tissues could be at least partially due to hormonal changes associated with the development or maintenance of the differentiated state.

Somewhat irrespective of the factors regulating glucanase expression, it is of interest to question the role of those enzymes. Considerable attention has been given to their participation in plant defense mechanisms. A defense role could be consistent with their synthesis in the roots of uninfected plants and possibly also in the older leaves at the base of the plant; these enzymes in combination with other defense proteins could represent a permanent system of protection against pathogens present in the rhizosphere. However, the selective expression in the stem cells at the bases of emerging shoots in the upper part of the plant strongly suggests that β-1,3-glucanases play an undefined role in plant development.

The Expression of β-1,3-Glucanases Is Differentially Regulated

Induction of PR proteins in healthy plants has been correlated with flowering (Fraser, 1981; Lotan et al., 1989). However, in our experiments, no apparent changes were observed in expression conferred by the *gn1* promoter during plant flowering. We have shown that the expression conferred by the *gn1* promoter was highly induced after salicylic acid treatment, whereas only a weak induction was obtained in response to ethylene and mechanical wounding. Ethylene has been shown to induce the expression and synthesis of some tobacco β-1,3-glucanases (Felix and Meins, 1987; Mauch and Staehelin, 1989; Memelink et al., 1990). In addition, the expression of a second

β-1,3-glucanase gene (*gn2*) from *N. plumbaginifolia* was highly induced after ethylene treatment (C. Castresana, unpublished data). In this context, the response observed in the expression conferred by the *gn1* promoter suggests that the β-1,3-glucanase isoforms would be differentially regulated in response to various stimuli.

As with ethylene, the level of salicylic acid in tobacco leaves increases during hypersensitive reaction (Klessig et al., 1989). In this respect, both salicylic acid and ethylene could play a role as cellular signals controlling the plant response against pathogen attack mediated through activation of at least partially independent transduction pathways.

The GN1 Protein Participates in the Plant Defense Response

We have shown for three plant-bacteria interactions that the expression conferred by the *gn1* promoter was highly induced only when infection resulted in a hypersensitive reaction. This induction of expression occurred locally, as shown by a strong GUS staining at the inoculation site surrounding the necrotic lesion. Similarly, the expression of various genes involved in plant defense responses has been shown to be localized around the infected area (Schmelzer et al., 1989; Ohshima et al., 1990). This spatial induction that we have characterized suggests that the β-1,3-glucanases, and probably other defense reactions, could act primarily as a barrier controlling the size of the lesion and the pathogen spread.

Induction of PR proteins has been shown to occur in certain compatible interactions. In this respect, we observed a threefold induction in the level of GUS activity in response to inoculation with the compatible pathogen bacterium *E. carotovora* subsp. *carotovora*. It is possible that the sevenfold higher induction corresponding to the incompatible interaction, in combination with similar differential induction levels for other genes, accounts for the resistance response. This observation seems even more plausible, given the strikingly localized expression observed from the *gn1* promoter. In this localized region, the difference in levels of expression distinguishing the compatible and incompatible interactions is likely to be much higher than the sevenfold difference we measured. The results support the possibility that the GN1 protein participates in plant defense mechanisms.

METHODS

Isolation and Characterization of *gn1*

The molecular characterization of the gene *gn1* was carried out using standard DNA procedures described by Maniatis et al. (1982). Plasmid vectors pUC18 and pGEM2 were used for DNA subcloning. DNA sequences were determined using the chain-termination method of Sanger et al. (1977).

Bacteriophage T7 RNA Polymerase Expression of the *gn1* Gene in *Escherichia coli*

The *gn1* cDNA lacking its signal sequence was inserted into the T7 RNA polymerase expression vector pT7-7. The recombinant plasmid pT7-7/*gn1* was transferred to *E. coli* strain K38 containing the T7 RNA polymerase gene under the control of the P1 promoter in the plasmid pGP1-2 (Tabor and Richardson, 1985). *E. coli* containing both plasmids were grown at 30°C in LB medium with 50 μg/mL carbenicillin and kanamycin to a cell density of 0.5 (A_{595}). Induction and labeling of the GN1 protein were carried out essentially as described by Tabor and Richardson (1985). Cells (0.2 mL) were centrifuged at 10,000*g* for 5 min and washed twice with 5 mL of M9 medium. The cell pellet was resuspended in 1 mL of M9 medium supplemented with 20 μg/mL thiamine and 0.001% amino acids (with the exception of cysteine and methionine) and incubated at 30°C for 60 min. Temperature was shifted at 42°C for 15 min and rifampicin was added to a final concentration of 200 μg/mL. After incubation for 10 additional min, the temperature was shifted down to 30°C for 20 min. Cells were pulsed with 10 μCi of ^{35}S-methionine for 5 min, centrifuged at 10,000*g* for 5 min, and resuspended in 100 μL of lysis buffer (12 mM Tris-HCl, pH 6.8, 1% SDS, 50 mM DTT, 10% glycerol, 0.1% bromophenol blue). Samples were heated to 95°C for 3 min and separated by SDS-PAGE according to Laemmli (1970) using 15% polyacrylamide gels. Gels were dried and analyzed by autoradiography.

Assay for β-1,3-Glucanase Enzyme Activity

To maximize protein production using the T7 RNA polymerase/promoter system, *E. coli* cells containing the two plasmids pT7-7/*gn1* and pGP1-2 were grown at 30°C in LB medium with 50 μg/mL carbenicillin and kanamycin to a cell density of 1.5 (A_{595}). The temperature was raised to 42°C for 25 min. After rifampicin addition (100 μg/mL final concentration), the temperature was shifted to 37°C for two additional hr. Cells were harvested by centrifugation at 10,000*g* for 5 min, resuspended in 1 mL of lysis buffer (12 mM Tris-HCl, pH 6.8, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), and disrupted by sonication. After centrifugation at 10,000*g* for 5 min, the β-1,3-glucanase activity was analyzed both in the supernatant and in the pellet fraction. Pellets were resuspended in 1 mL of lysis buffer with 8 M urea, incubated 10 min at 25°C, and centrifuged at 12,000*g* for 5 min. Solubilized proteins were extensively dialyzed against 12 mM Tris-HCl (pH 6.8), 1 mM EDTA. β-1,3-Glucanase activity was assayed by detection of reducing sugars released after incubation with the substrate laminarin, using the method described by Somogyi (1952) and Nelson (1957).

Analysis of *gn1* Expression

RNA was prepared according to the procedure described by Jones et al. (1985). For RNA gel blot experiments, 10 μg of total

RNA were separated in formaldehyde gels as described by Maniatis et al. (1982). The cDNA from the *gn1* gene cloned in the pGEM2 vector (De Loose et al., 1988) was used to prepare a single-stranded riboprobe using the Amersham transcription kit. Blots were hybridized overnight at 68°C in 50% formamide, 3 × SSC, 0.25% nonfat milk powder, 0.5% SDS, and 20 μg/mL denatured herring sperm DNA. The hybridizations were washed at 68°C twice with 3 × SSC, 1% SDS and twice with 1 × SSC, 1% SDS for 30 min.

Primer extension analyses were carried out essentially as described by Ausubel et al. (1988).

Agrobacterium-Mediated DNA Transfer and Analysis of Transgenic Plants

The vector pDE1001 containing the *gn1*/GUS chimeric construct was mobilized by the helper plasmid pRK2013 (Figurski and Helinski, 1979) to *Agrobacterium tumefaciens* harboring the plasmid pGV2260 (Deblaere et al., 1985). The chimeric construct was transferred to *Nicotiana tabacum* SR1 cells by leaf disc transformation, and the transgenic plants were selected on kanamycin-containing medium (Horsch et al., 1985). Approximately 12 weeks after transfer to soil, the transgenic plants were examined. The integrity and number of T-DNA copies inserted into the plant genome were estimated by DNA gel blot (Southern, 1975), using DNA isolated according to the method described by Dellaporta et al. (1983).

GUS activity was measured according to the fluorimetric assay described by Jefferson et al. (1987). Protein concentration was determined by the method of Bradford (1976) using a kit supplied by Bio-Rad Laboratories. The histochemical GUS assay was carried out as described by Peleman et al. (1989).

Expression of *gn1* in Response to Plant Stress

GUS activity was assayed in leaves corresponding to the upper part of the transgenic plants before and after the following stress treatments: For salicylic acid induction, plants were sprayed with a 5 mM solution of this compound; the effect of ethylene was examined by placing the plants in glass jars and flushing continuously at a rate of 100 mL/min with 50 ppm ethylene. For wounding experiments, leaves were extensively punctured using a needle. Bacterial infection was done by injection of the leaves with a late-logarithmic culture. The bacteria were grown in LPG medium (0.3% yeast extract, 0.5% Bacto-peptone, and 0.5% glucose) at 27°C. After centrifugation, the pellets were resuspended in sterile, distilled water to reach a final concentration of 10^7 bacteria/mL. The following bacterial species were used: *Pseudomonas syringae* pv *syringae* (NCPPB281), *Erwinia carotovora* subsp. *carotovora* (NCPPB550), and *Pseudomonas fluorescens* (ATCC13525). GUS activity was assayed in at least 10 independent transgenic plants 48 hr after each treatment. In addition, wound inducibility was evaluated at 5 hr, 10 hr, and 24 hr after mechanical stress.

ACKNOWLEDGMENTS

The T7 RNA polymerase promoter strains were a kind gift of Dr. Stan Taylor (Harvard University). We thank Allan Caplan, Marc Van den Bulcke, and Alfredo Herrera-Estrella for helpful discussions and critical reading; Nancy Terryn for helping in subcloning the *gn1* λ clone; Kris Genetello, Jan Gielen, and Willy De Keyzer for expert technical assistance; and Martine De Cock and Vera Vermaercke for preparing the manuscript and diagrams. This work was supported by grants from the Algemene Spaar- en Lijfrentekas-Kankerfonds and the Services of the Prime Minister (U.I.A.P. 120C0187). C.C. was supported by an EMBO long-term fellowship and F.d.C. by a Brazilian Education Agency (CAPES) postgraduate fellowship; D.I. is a senior research assistant of the National Fund for Scientific Research (Belgium).

Received July 25, 1990; accepted September 25, 1990.

REFERENCES

An, G., Costa, M.A., Mitra, A., Ha, S.-H., and Márton, L. (1988). Organ-specific and developmental regulation of the nopaline synthase promoter in transgenic tobacco plants. Plant Physiol. **88,** 547–552.

An, G., Costa, M.A., and Ha, S.-B. (1990). Nopaline synthase promoter is wound inducible and auxin inducible. Plant Cell **2,** 225–233.

Antoniw, J.F., Ritter, C.E., Pierpoint, W.S., and van Loon, L.C. (1980). Comparison of three pathogenesis-related proteins from plants of two cultivars of tobacco infected with TMV. J. Gen. Virol. **47,** 79–87.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds (1988). Current Protocols in Molecular Biology 1987–1988. (New York: John Wiley & Sons).

Bol, J.F., Hooft van Huijsduijnen, R.A.M., Cornelissen, B.J.C., and van Kan, J.A.L. (1987). Characterization of pathogenesis-related proteins and genes. In Plant Resistance to Viruses (Ciba Foundation Symposium 133), D. Evered and S. Harnett, eds (Chichester: John Wiley & Sons), pp. 72–91.

Boller, T. (1987). Hydrolytic enzymes in plant disease resistance. In Plant-Microbe Interactions, Vol. 2, T. Kosuge and E.W. Nester, eds (New York: MacMillan), pp. 385–413.

Boller, T., and Vögeli, U. (1984). Vacuolar localization of ethylene-induced chitinase in bean leaves. Plant Physiol. **74,** 442–444.

Boller, T., Gehri, A., Mauch, F., and Vögeli, U. (1983). Chitinase in bean leaves: Induction by ethylene, purification, properties, and possible function. Planta **157,** 22–31.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Broglie, K.E., Gaynor, J.J., and Broglie, R.M. (1986). Ethylene regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. Proc. Natl. Acad. Sci. USA **83,** 6820–6824.

Campbell, R., and Greaves, M.P. (1990). Anatomy and community structure of the rhizosphere. In The Rhizosphere, J.M. Lynch, ed (Chichester: John Wiley & Sons), pp. 11–34.

Carr, J.P., and Klessig, D.F. (1990). The pathogenesis-related

proteins of plants. In Genetic Engineering, Principles and Methods, Vol. 11, J.K. Setlow, ed (New York: Plenum Press), pp. 65–109.

Collinge, D.B., and Slusarenko, A.J. (1987). Plant gene expression in response to pathogens. Plant Mol. Biol. **9,** 389–410.

Deblaere, R., Bytebier, B., De Greve, H., Deboeck, F., Schell, J., Van Montagu, M., and Leemans, J. (1985). Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. Nucl. Acids Res. **13,** 4777–4788.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. **1,** 19–21.

De Loose, M., Alliotte, T., Gheysen, G., Genetello, C., Gielen, J., Soetaert, P., Van Montagu, M., and Inzé, D. (1988). Primary structure of a hormonally regulated β-glucanase of *Nicotiana plumbaginifolia*. Gene **70,** 13–23.

Ecker, J.R., and Davis, R.W. (1987). Plant defense genes are regulated by ethylene. Proc. Natl. Acad. Sci. USA **84,** 5202–5206.

Evered, D., and Harnett, S., eds (1987). Plant Resistance to Viruses (Ciba Foundation Symposium 133) (Chichester: John Wiley & Sons).

Felix, G., and Meins, F., Jr. (1986). Developmental and hormonal regulation of β-1,3-glucanase in tobacco. Planta **167,** 206–211.

Felix, G., and Meins, F., Jr. (1987). Ethylene regulation of β(1,3)-glucanase in tobacco. Planta **172,** 386–392.

Figurski, D.H., and Helinski, D.R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76,** 1648–1652.

Fraser, R.S.S. (1981). Evidence for the occurrence of the "pathogenesis-related" proteins in leaves of healthy tobacco plants during flowering. Physiol. Plant Pathol. **19,** 69–76.

Fritig, B., Kauffmann, S., Dumas, B., Geoffroy, P., Kopp, M., and Legrand, M. (1987). Mechanism of the hypersensitivity reaction of plants. In Plant Resistance to Viruses (Ciba Foundation Symposium 133), D. Evered and S. Harnett, eds (Chichester: John Wiley & Sons), pp. 92–108.

Gheysen, G., Inzé, D. Soetaert, P., Van Montagu, M., and Castresana, C. (1990). Sequence of a *Nicotiana plumbaginifolia* β-1,3-glucanase gene encoding a vascular isoform. Nucl. Acids Res. **22,** in press.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science **227,** 1229–1231.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: β-Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. **6,** 3901–3907.

Jones, J.D.G., Dunsmuir, P., and Bedbrook, J. (1985). High level expression of introduced chimeric genes in regenerated transformed plants. EMBO J. **4,** 2411–2418.

Joosten, M.H.A.J., and De Wit, J.G.M. (1989). Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as 1,3-β-glucanases and chitinases. Plant Physiol. **89,** 945–951.

Kauffmann, S., Legrand, M., Geoffroy, P., and Fritig, B. (1987). Biological function of "pathogenesis-related" proteins: Four PR proteins of tobacco have 1,3-β-glucanase activity. EMBO J. **6,** 3209–3212.

Keen, N.T., and Yoshikawa, M. (1983). β-1,3-Endoglucanase from soybean releases elicitor-active carbohydrates from fungus cell walls. Plant Physiol. **71,** 460–465.

Keil, M., Sanchez-Serrano, J.J., and Willmitzer, L. (1989). Both wound-inducible and tuber-specific expression are mediated by the promoter of a single member of the potato proteinase inhibitor II gene family. EMBO J. **8,** 1323–1330.

Keller, B., Schmid, J., and Lamb, C.J. (1989). Vascular expression of a bean cell wall glycine-rich protein-β-glucuronidase gene fusion in transgenic tobacco. EMBO J. **8,** 1309–1314.

Klessig, D.F., Cutt, J.R., Dixon, D.C., Carr, J.P., Malamy, J., Metzler, M., Harpster, M.H., Dunsmuir, P., Beachy, R.N., and Raskin, I. (1989). The PR1 proteins—Their synthesis, location, and possible function. Abstract presented at the Fundacion Juan March International Meeting on "Pathogenesis-Related Proteins in Plants," October 23–25, 1989, Valencia, Spain.

Kombrink, E., Schröder, M., and Hahlbrock, K. (1988). Several "pathogenesis-related" proteins in potato are 1,3-β-glucanases and chitinases. Proc. Natl. Acad. Sci. USA **85,** 782–786.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Langridge, W.H.R., Fitzgerald, K.J., Koncz, C., Schell, J., and Szalay, A.A. (1989). Dual promoter of *Agrobacterium tumefaciens* mannopine synthase genes is regulated by plant growth hormones. Proc. Natl. Acad. Sci. USA **86,** 3219–3223.

Lotan, T., Ori, N., and Fluhr, R. (1989). Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell **1,** 881–887.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Mauch, F., and Staehelin, L.A. (1989). Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell **1,** 447–457.

Mauch, F., Mauch-Mani, B., and Boller, T. (1988a). Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. **88,** 936–942.

Mauch, F., Hadwiger, L.A., and Boller, T. (1988b). Antifungal hydrolases in pea tissue. I. Purification and characterization of two chitinases and two β-1,3-glucanases differentially regulated during development and in response to fungal infection. Plant Physiol. **87,** 325–333.

Memelink, J., Hoge, J.H.C., and Schilperoort, R.A. (1987). Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco. EMBO J. **6,** 3579–3583.

Memelink, J., Linthorst, H.J.M., Schilperoort, R.A., and Hoge, J.H.C. (1990). Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. Plant Mol. Biol. **14,** 119–126.

Mohnen, D., Shinshi, H., Felix, G., and Meins, F., Jr. (1985). Hormonal regulation of β-1,3-glucanase messenger RNA levels in cultured tobacco tissue. EMBO J. **4,** 1631–1635.

Moore, T.C. (1989). Biochemistry and Physiology of Plant Hormones, 2nd ed. (New York: Springer-Verlag).

Nelson, N.J. (1957). Colorimetric analysis of sugars. Methods Enzymol. **3,** 85–86.

Ohshima, M., Itoh, H., Matsuoka, M., Murakami, T., and Ohashi, Y. (1990). Analysis of stress-induced or salicylic acid-induced expression of the pathogenesis-related 1a protein gene in transgenic tobacco. Plant Cell **2,** 95–106.

Peleman, J., Boerjan, W., Engler, G., Seurinck, J., Botterman, J., Alliotte, T., Van Montagu, M., and Inzé, D. (1989). Strong cellular preference in the expression of a housekeeping gene of *Arabidopsis thaliana* encoding *S*-adenosylmethionine synthetase. Plant Cell **1,** 81–93.

Poulsen, C., Fluhr, R., Kauffman, J.M., Boutry, M., and Chua, N.-H. (1986). Characterization of an *rbcS* gene from *Nicotiana plumbaginifolia* and expression of an *rbcS*-CAT chimeric gene in homologous and heterologous nuclear background. Mol. Gen. Genet. **205,** 193–200.

Rigden, J., and Coutts, R. (1988). Pathogenesis-related proteins in plants. Trends Genet. **4,** 87–89.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Schmelzer, E., Krüger-Lebus, S., and Hahlbrock, K. (1989). Temporal and spatial patterns of gene expression around sites of attempted fungal infection in parsley leaves. Plant Cell **1,** 993–1001.

Shinshi, H., Mohnen, D., and Meins, F., Jr. (1987). Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc. Natl. Acad. Sci. USA **84,** 89–93.

Shinshi, H., Wenzler, H., Neuhaus, J.-M., Felix, G., Hofsteenge, J., and Meins, F., Jr. (1988). Evidence for N- and C-terminal processing of a plant defense-related enzyme: Primary structure of tobacco prepro-β-1,3-glucanase. Proc. Natl. Acad. Sci. USA **85,** 5541–5545.

Somogyi, M. (1952). Notes on sugar determination. J. Biol. Chem. **195,** 19–23.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98,** 503–517.

Stanford, A.C., Northcote, D.H., and Bevan, M.W. (1990). Spatial and temporal patterns of transcription of a wound-induced gene in potato. EMBO J. **9,** 593–603.

Tabor, S., and Richardson, C.C. (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82,** 1074–1078.

Van de Rhee, M.D., Van Kan, J.A.L., González-Jaén, M.T., and Bol, J.F. (1990). Analysis of regulatory elements involved in the induction of two tobacco genes by salicylate treatment and virus infection. Plant Cell **2,** 357–366.

Van den Bulcke, M., Bauw, G., Castresana, C., Van Montagu, M., and Vandekerckhove, J. (1989). Characterization of vacuolar and extracellular β(1,3)-glucanases of tobacco: Evidence for a strictly compartmentalized plant defense system. Proc. Natl. Acad. Sci. USA **86,** 2673–2677.

van Loon, L.C. (1985). Pathogenesis-related proteins. Plant Mol. Biol. **4,** 111–116.

van Loon, L.C., and van Kammen, A. (1970). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. "Samsun" and "Samsun NN." II. Changes in protein constitution after infection with tobacco mosaic virus. Virology **40,** 199–211.

van Loon, L.C., Gerritsen, Y.A.M., and Ritter, C.E. (1987). Identification, purification and characterization of pathogenesis-related proteins from virus-infected Samsun NN tobacco leaves. Plant Mol. Biol. **9,** 593–609.

Vögeli, U., Meins, F., Jr., and Boller, T. (1988). Co-ordinated regulation of chitinase and β-1,3-glucanase in bean leaves. Planta **174,** 364–372.

Vögeli-Lange, R., Hansen-Gehri, A., Boller, T., and Meins, F., Jr. (1988). Induction of the defense-related glucanohydrolases, β-1,3-glucanase and chitinase, by tobacco mosaic virus infection of tobacco leaves. Plant Sci. **54,** 171–176.

White, R.F. (1979). Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. Virology **99,** 410–412.

Yang, S.F., and Hoffman, N.E. (1984). Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. **35,** 155–189.