

# Functional Analysis of DNA Sequences Responsible for Ethylene Regulation of a Bean Chitinase Gene in Transgenic Tobacco

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Expression of at least two genes from bean encoding the defense-related protein chitinase has been shown previously to be transcriptionally regulated by the phytohormone ethylene. We have determined the complete nucleotide sequence of one of these genes, the CH5B gene, which resides on a 4.7-kilobase fragment of bean genomic DNA. The structural gene consists of a single open reading frame and encodes the 301 amino acids of the mature protein and a 26-amino acid signal peptide. The CH5B gene has been introduced into tobacco plants using *Agrobacterium* Ti-plasmid vectors. Little or no expression of the bean gene was observed when transgenic tobacco plants were grown in air; however, exposure of these plants to an atmosphere containing 50 parts per million ethylene resulted in an approximately 20-fold to 50-fold increase in the level of the bean chitinase mRNA. Ethylene-dependent expression of a chimeric gene consisting of 1.6 kilobases of 5'-flanking DNA derived from the CH5B gene fused to the coding sequence of  $\beta$ -glucuronidase indicates that this region of the CH5B gene is sufficient for ethylene-regulated expression. Deletion analysis of the CH5B promoter region has allowed us to localize these DNA sequences to within a 228-base pair region situated between -422 and -195 upstream of the transcriptional start site. This region is characterized by two short DNA sequences that are exactly conserved in a second ethylene-regulated bean chitinase gene.

## INTRODUCTION

Ethylene is an endogenous plant hormone that influences many aspects of plant growth and development. Normal physiological processes such as seed germination, seedling growth, fruit ripening, and plant senescence are all subject to regulation by this phytohormone (Abeles, 1973). Ethylene is also known to function during conditions of environmental stress. Increased biosynthesis of this plant hormone has been shown to accompany oxygen deficiency, flooding (Metraux and Kende, 1983; Raskin and Kende, 1984), wounding (Boller and Kende, 1980), and pathogen attack (Toppan, Roby, and Esquerre-Tugay, 1982). In the case of bacterial or fungal infection, ethylene production has been correlated with the induction of several proteins that presumably function in the defense response of the plant. Among these are the hydroxyproline-rich glycoproteins (Roby, Toppan, and Esquerre-Tugay, 1985),  $\beta$ -1,3-glucanase (Felix and Meins, 1987), and chitinase (Vögeli, Meins, and Boller, 1988).

The endochitinase from bean is a 30-kD protein that catalyzes the hydrolysis of chitin, a  $\beta$ -1,4-linked homopolymer of *N*-acetyl-D-glucosamine (Boller et al., 1983). Al-

though no endogenous substrate for the enzyme is known to exist in higher plants, chitin is a ubiquitous component of the cell walls of most fungi (Wessels and Sietsma, 1981). For this reason, it has been suggested that chitinase functions as a defense against chitin-containing pathogens (Abeles et al., 1970). In vitro studies with the purified enzyme from bean and tomato have provided some evidence in support of this hypothesis (Young and Pegg, 1982; Boller et al., 1983; Schlumbaum et al., 1986).

In healthy, uninfected plants, chitinase levels are low or undetectable. However, an induction of enzyme activity is observed upon treatment with ethylene or elicitors or upon infection with fungal pathogens (Boller, 1985). Full-length cDNA clones encoding an ethylene-induced bean chitinase have been isolated and shown to encode a protein of *M*<sub>r</sub> 35,400 (Broglie, Gaynor, and Broglie, 1986). The 984-bp open reading frame specifies the entire 301 amino acids of the mature protein and a 27-residue amino-terminal sequence, which presumably functions in determining the vacuolar localization of the bean enzyme (Boller and Vögeli, 1984). DNA gel blot analyses indicate that, in bean, chitinase is encoded by a small, multigene family consisting of at least three members. Using one of these cDNA clones as a hybridization probe, three chitinase genes have been

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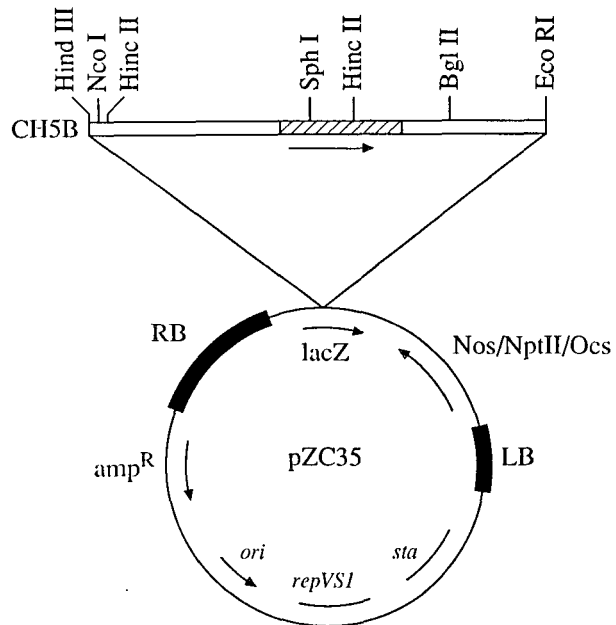
an isoleucine and the other results in the conversion of a tryptophan to a cysteine residue.

The transcriptional start site of the chitinase mRNA was determined by S1 nuclease mapping using a 5' end-labeled Sau3A fragment comprising nucleotides -134 to +245. Through this analysis, the predominant 5' terminus was localized to the C residue which is found 25 nucleotides upstream of the initiator ATG codon. A putative TATA box is found at position -28 and a CAAT-like sequence resides at -182. At the 3' end of the gene, consensus sequences for polyadenylation are found at +1027, +1031, and +1101. Data obtained from nucleotide sequence analysis of chitinase cDNA clones indicate that both the upstream and the downstream polyadenylation sites are utilized with preferential poly(A) addition occurring at position +1121.

### Expression of the Chitinase CH5B Gene in Transgenic Tobacco Is Ethylene-Dependent

The HindIII-EcoRI fragment containing the chitinase CH5B gene was introduced into tobacco by *Agrobacterium tumefaciens*-mediated transformation. The 4.7-kb restriction fragment was first cloned into the polylinker region of the binary vectors pZS762 and pZS761 to yield pZC35 and pZC135, respectively, as shown in Figure 2. pZS762 contains a left border fragment of the octopine Ti plasmid pTiA6, and a right border fragment derived from pTiAch5 (van den Elzen et al., 1985). Between the left and right border fragments is positioned the polylinker sequence of pUC19 and a chimeric marker gene (NOS/NPTII/OCS) that specifies kanamycin resistance in plant cells. The amp<sup>R</sup> segment provides ampicillin resistance in *Escherichia coli* and *A. tumefaciens*. pZS761 is essentially identical to pZS762 except that the orientation of the polylinker segment is reversed. The recombinant plasmids containing the chitinase CH5B gene were introduced into *A. tumefaciens* strain LBA 4404 by bacterial conjugation using a three-way mating procedure (Ruvkin and Ausubel, 1981). The *Agrobacterium* samples were then used for infection of tobacco leaf discs (Horsch et al., 1985).

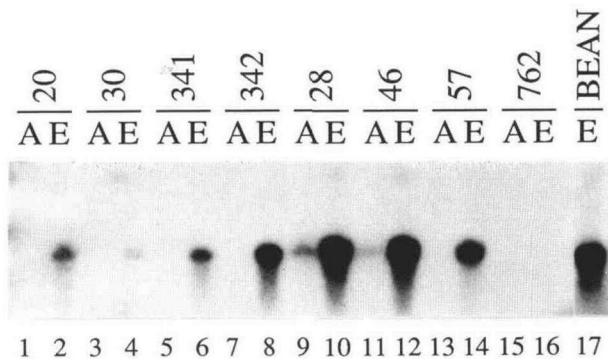
To assay the expression of the CH5B gene in the heterologous plants, RNA gel blot analyses were performed. Total leaf RNA was isolated from individual transgenic tobacco plants harboring either the control plasmid pZS762, or pZC35 or pZC135. Figure 3 shows that, in each case, the level of hybridization of chitinase mRNA increases when RNA is isolated from plants exposed to 50 ppm ethylene. In contrast, little or no signal is detected when RNA, isolated from pZC35 or pZC135 transformants grown in the presence of air alone, is hybridized to nick-translated cDNA clone pCH18. RNA from untreated or ethylene-treated plants containing only pZS762 binary vector sequences also fails to hybridize to the bean chitinase cDNA clone, indicating that, under these hybridization conditions, endogenous tobacco chitinase mRNA is not detected.



**Figure 2.** Structure of the Intermediate Binary Vectors pZC35 and pZC135 Containing the 4.7-kb Fragment of Bean Genomic DNA.

The 4.7-kb HindIII-EcoRI fragment containing the CH5B gene was cloned into the polylinker region of the binary vectors pZS761 and pZS762 to create pZC35 and pZC135, respectively. RB, right border, derived from Ti plasmid pTiAch5; LB, left border, derived from the octopine Ti plasmid pTiA6; amp<sup>R</sup>, ampicillin resistance; *rep* and *sta*, regions of *Pseudomonas aeruginosa* plasmid pSV1 essential for replication and stable maintenance, respectively, in *A. tumefaciens* (Itoh et al., 1984); pNOS, promoter fragment derived from the nopaline synthase gene; NptII, coding region for neomycin phosphotransferase; OCS 3',3'-untranslated region of octopine synthase.

Although we found the intensity of the hybridizing band to vary among different transformants, in general, a somewhat higher level of expression was often seen in plants carrying the pZC35 construct. This difference may be due to the presence of the OCS activator element in the right border fragment of the binary vector, which, in this construct, is situated upstream of the chitinase gene. A position-dependent increase in steady-state mRNA levels attributable to an OCS activator effect has previously been noted by Taylor et al. (1987). In the case of maize ADHI, cloning of the gene downstream from the OCS activator resulted in an increased transcriptional efficiency sufficient to render the monocot gene detectable in transgenic tobacco plants while still maintaining anaerobic regulation of ADHI expression (Ellis et al., 1987). Similarly, for the chitinase CH5B gene, overall mRNA levels in plants containing the pZC35 construct are somewhat higher than in the



**Figure 3.** Expression of Bean Chitinase in Transgenic Tobacco.

RNA gel blots of total RNA isolated from untreated and ethylene-treated transgenic tobacco were probed with labeled insert from bean chitinase cDNA clone pCH18. Lanes 1 to 8, 24  $\mu$ g of total RNA from individual pZC135 transformants grown in air alone (A) or following treatment with air containing 50 ppm ethylene (E) for 36 hr; lanes 9 to 14, 24  $\mu$ g of total RNA extracted from individual pZC35 transformants grown as described above.

pZC135 transformants; however, in both cases, ethylene-regulated expression can be demonstrated. In addition, the size of the RNA transcript detected in both instances is identical to that in ethylene-treated bean plants. Together, these results suggest that transcription of the bean chitinase gene in transgenic tobacco plants is from its own promoter rather than from sequences located elsewhere in the vector and that the sequences responsible for ethylene-regulated expression of the bean CH5B gene are contained within the 4.7-kb HindIII-EcoRI fragment of pZC35 and pZC135. Moreover, the required transcriptional factors are present in tobacco and are able to recognize the signals in the CH5B gene to provide accurate initiation of mRNA synthesis in the presence of ethylene.

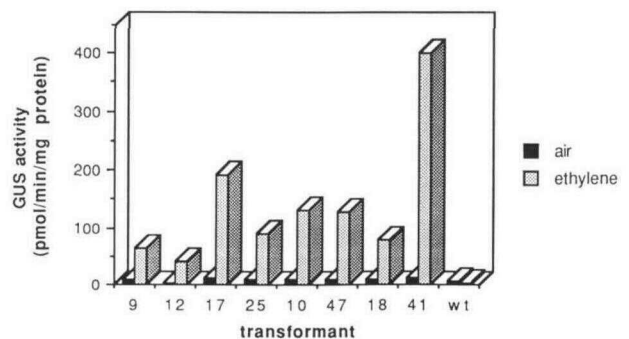
### 5' Upstream Sequences Are Responsible for Ethylene Induction of Gene Expression

As a first step in defining the DNA sequence elements responsible for ethylene regulation of chitinase expression, we constructed a chimeric gene by fusing DNA fragments derived from the upstream region of the CH5B gene to the coding sequence of  $\beta$ -glucuronidase (GUS). Chitinase promoter fragments were obtained by Bal31 digestion of SphI-linearized pCH35 and characterized by DNA sequence analysis. Two promoter fragments with 3' endpoints located at +27 and +50 were ligated to GUS structural sequences contained within the binary vectors pBI101.1 and pBI101.2, respectively. The polyadenylation signals were provided by a 260-bp nopaline synthase 3' end fragment. The resultant recombinant plasmids, pCG1223

and pCG2226, were introduced into *A. tumefaciens* LBA 4404 and used to infect tobacco leaf discs.

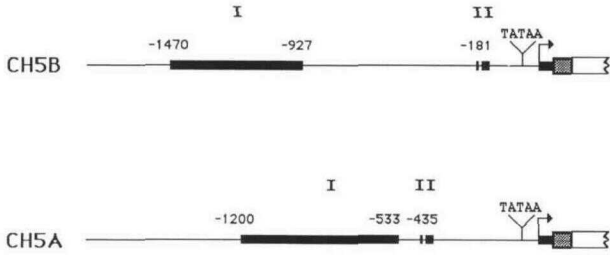
Regenerated tobacco plants harboring either the pCG1223 or the pCG2226 construct were assayed for ethylene induction of GUS enzyme activity as described in Methods. A summary of the assay data is given in Figure 4. Transgenic plants containing pCG1223 show a fivefold to 18-fold induction of GUS activity upon exposure to ethylene, with the average increase in enzyme activity being approximately 10-fold. Transformants containing pCG2226 undergo an eightfold to 30-fold induction, giving rise to an average 16-fold induction of GUS activity with ethylene treatment. Negligible GUS activity is found in untreated or treated control plants, which lack the chimeric GUS construct. The GUS activity data allow us to conclude that sequences upstream of the chitinase coding region between  $-1651$  and  $+27$  are sufficient to specify ethylene-regulated expression of the CH5B gene.

Previous studies have shown that at least two of the three chitinase genes known to be present in the bean genome are subject to regulation by the phytohormone, ethylene (Broglie, Gaynor, and Broglie, 1986). To identify conserved regions that may be important in mediating this response, we compared the DNA sequence of the upstream region of the CH5B gene to that of a 1500-bp segment of 5'-flanking DNA that precedes the structural region of the CH5A gene. Although the upstream regions of the two genes are for the most part divergent, two conserved regions of sequence homology are found. These are shown in Figure 5. Region I is located between  $-927$  and  $-1470$  in the CH5B gene. This DNA segment is AT-rich and shows 96% homology to the corresponding region in the CH5A gene. Region II consists of two



**Figure 4.** Comparison of  $\beta$ -Glucuronidase Enzymatic Activity in Untreated and Ethylene-Treated Transgenic Tobacco.

Individual transformants containing either pCG1223 or pCG2226 were assayed for GUS activity, as described in Methods, after growth in air or following a 36-hr treatment with air containing 50 ppm ethylene. Plants 9, 12, 17, and 25 were transformed with pCG1223, and plants 10, 47, 18, and 41 contain pCG2226. Controls were untransformed tobacco plants (wt).



**Figure 5.** Schematic Diagram of 5' Upstream Regions of CH5B and CH5A Genes.

Comparison of the nucleotide sequences of 2.0 kb and 1.5 kb of 5' flanking regions of the CH5B and CH5A genes, respectively. Nucleotide sequence analysis was performed on both strands. Sequences were aligned using the BestFit program of the University of Wisconsin sequence analysis software package. Bold lines indicate regions of conserved sequence homology.

stretches of 13 bp and 43 bp that are perfectly conserved between both chitinase genes. In the CH5B gene, these segments occur at -181 to -223 and -252 to -264, whereas in the CH5A gene, they reside further upstream, between -435 and -477 and -494 and -505.

To evaluate the significance of these regions of homology shared by the CH5A and CH5B genes, and as a first step toward the identification of DNA sequences required for the ethylene regulation of chitinase gene expression, a series of promoter deletion mutants was created. The 5'-flanking region of the CH5B gene was modified as described in Methods, and the shortened genes were cloned into binary Ti plasmid DNA vectors. After conjugation into *A. tumefaciens* LBA 4404, the constructs were introduced into tobacco by inoculation of leaf discs with the *Agrobacterium* strains.

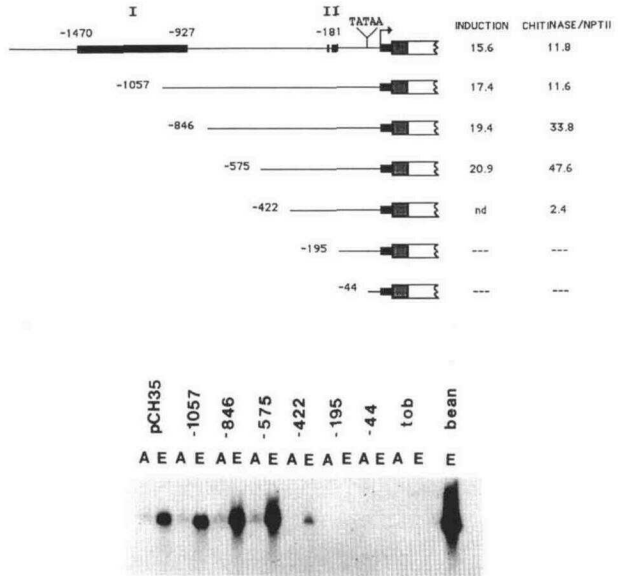
To assay the effect of the promoter deletions on ethylene-specific transcription of the CH5B gene, total RNA was isolated from transgenic plants before and after exposure to 50 ppm ethylene. A partial averaging of the effects of chromosomal insertion on mRNA levels was achieved by pooling equivalent amounts of RNA from six to eight individual transformants. After fractionation by agarose gel electrophoresis and transfer to nitrocellulose, RNA samples were hybridized with labeled pCH18 or NPTII DNA. RNA signals were quantitated by densitometric scanning of autoradiographic films. Chitinase mRNA levels are expressed relative to that of NPTII RNA in the transgenic plants.

Figure 6 shows an RNA gel blot of total RNA isolated from transgenic tobacco harboring the deleted chitinase genes and probed with pCH18 insert DNA. Region I does not appear to be necessary for ethylene regulation since deletion to -575 results in no significant loss in mRNA induction in response to ethylene treatment. The approximately threefold increase in the intensity of the hybridizing band of the -846 sample may indicate the removal of a

negative sequence element between -1057 and -846. Deletion to -422 results in an approximately 20-fold reduction in the levels of bean chitinase mRNA; however, induction by ethylene is still evidenced. Removal of upstream sequences to -195 results in a complete loss of the chitinase mRNA signal on RNA gel blots. Thus, DNA sequences located within this 228-bp segment appear to be crucial for ethylene induction of the chitinase CH5B gene. It is of some interest that the -195 deletion endpoint lies within the DNA region that is perfectly conserved between the CH5A and the CH5B genes (region II).

**DISCUSSION**

Chitinase gene expression has been shown to be regulated by a number of different stimuli, including wounding (Hed-



**Figure 6.** Activities of Various 5' Deletions of the CH5B Promoter.

(Upper) Diagrammatic representation of Bal31 deletions of CH5B. I and II correspond to conserved regions of sequence homology with the CH5A gene. The endpoints of the deletions are indicated on the left and are numbered from the transcriptional start site (+1). The -fold induction of chitinase mRNA in response to ethylene is indicated on the right. The chitinase/nptII ratios are also indicated on the right and were determined on samples derived from ethylene-treated tissue.

(Lower) RNA gel blot analysis of promoter deletions in transgenic tobacco. Lanes contain the following RNA samples: Lanes 1 to 16, equal amounts of total RNA (24 μg total per lane) from six to eight individual transformants containing wild-type or deleted CH5B genes; lanes 15 and 16, tobacco transformed with pZS762; lane 17, 3 μg of total bean RNA isolated from ethylene-treated plants. Hybridization was with labeled pCH18 insert (4 × 10<sup>8</sup> cpm/μg). A, air; E, ethylene.

rick et al., 1988), pathogen infection (Roby and Esquerre-Tugaye, 1987; Hedrick et al., 1988), treatment with fungal elicitor (Hedrick et al., 1988), and ethylene (Broglie, Gaynor, and Broglie, 1986). A corresponding increase in chitinase enzyme accumulation has also been observed immunologically and by enzymatic activity. In this paper, we have examined the *cis*-acting elements that are responsible for ethylene regulation of the bean chitinase CH5B gene. This gene was isolated on a 4.7-kb fragment of bean genomic DNA and is encoded by a single exon. When this fragment was introduced into tobacco, expression of the CH5B gene was found to be modulated by exogenous ethylene; levels of CH5B mRNA were 20-fold to 40-fold higher in primary transformants treated with ethylene than in untreated plants. Thus, all of the DNA sequences necessary for regulation of gene expression by this phytohormone appear to be contained within this DNA fragment. These data also suggest that the required transcription factors are conserved in tobacco and are able to recognize the regulatory elements of the heterologous bean gene to provide accurate induction of gene expression in response to ethylene treatment.

DNA sequences involved in the ethylene-regulated expression of CH5B were shown to be contained entirely within a 1.6-kb fragment situated immediately upstream of the chitinase coding sequences. This conclusion is based on an analysis of eight independent transgenic plants containing a chimeric gene composed of a fusion between 5'-flanking sequences of CH5B and the coding region of  $\beta$ -glucuronidase linked to a 3' fragment from nopaline synthase. As much as a 30-fold increase in GUS activity was observed in the presence of ethylene when under the control of the CH5B promoter. To define more precisely the regions of the CH5B promoter involved in ethylene induction of gene expression, a series of 5' deletion mutants was created in the CH5B promoter. These deletions were chosen after comparison of the upstream DNA sequences of two ethylene-regulated chitinase genes, CH5B and CH5A, and were analyzed in transgenic plants. Two strongly conserved regions of sequence homology were observed, and the deletions were chosen to remove selectively parts or all of these regions. Our results indicate that region I is not necessary for ethylene induction and may have a negative effect on the levels of gene expression. Additionally, when the promoter is deleted to -422, ethylene induction of gene expression is still observed, although mRNA levels are approximately 20-fold lower than for deletions to -575. This may result from the deletion of an activator sequence located between these two deletion endpoints. Similar results are obtained when CH5B promoter deletions fused to the GUS reporter gene are assayed in an ethylene-responsive bean transient expression system (D. Roby, K.E. Broglie, J.J. Gaynor, and R. Broglie, manuscript in preparation). A complete loss of ethylene-inducible gene expression is observed when DNA se-

quences are removed to -195, even though the remaining fragment still contains putative TATA and CAAT sequences. Interestingly, the -195 deletion endpoint lies within region II, which is perfectly conserved between the CH5A and CH5B genes. Thus, 5'-flanking sequences located within this 228-bp segment appear to be crucial for ethylene induction of CH5B gene expression.

Recently, 5'-flanking sequences of an ethylene-responsive tomato polygalacturonase gene have been shown to direct ripening-specific expression of a chimeric gene in transgenic tomato (Bird et al., 1988). Deikman and Fischer (1988) have identified DNA binding factors that interact with 5'-flanking sequences of two additional ethylene-responsive fruit-ripening genes from tomato. It will be of interest to determine whether the *cis*-acting DNA sequence(s) in the CH5B gene also bind specific *trans*-acting factors.

In addition to ethylene, chitinase gene expression can be achieved by wounding, treatment with fungal elicitor, and pathogen infection. However, the relationship among these various stimuli (Mauch, Hadwiger, and Boller, 1984) and the nature of the signal/transduction pathway are not well understood. Recently, it has been reported that treatment of cell suspension cultures (Chappell, Hahlbrock, and Boller, 1984; Tong, Labavitch, and Yang, 1986) or melon plants (Roby, Toppan, and Esquerre-Tugaye, 1985) with fungal elicitors results in rapid increases in ethylene production. In this regard, it would be of interest to determine whether the same *cis*-acting elements and *trans*-acting factors modulate chitinase gene expression in response to these various stresses.

## METHODS

### Isolation and Characterization of Bean Chitinase Genes

Chitinase genomic clones were isolated from an Sau3A library of bean genomic DNA in the vector EMBL4 (Broglie, Gaynor, and Broglie, 1986). Purified clones were characterized by restriction enzyme mapping and DNA gel blot analysis (Southern, 1975) to identify DNA segments harboring the chitinase genes. The 4.7-kb HindIII-EcoRI fragment of  $\lambda$ CH5B was isolated, rendered blunt-ended by treatment with the Klenow fragment of DNA polymerase I, and subcloned in both orientations into the SmaI site of pEMBL8+. The resultant plasmids are termed pCH34 and pCH35. A 1.7-kb fragment of  $\lambda$ CH5A, which contained approximately 200 bp of coding region and 1500 bp of 5'-flanking DNA, was blunt-ended and also cloned into the SmaI site of pEMBL8+. These recombinant plasmids are designated pCH39 and pCH40. Subclones pCH34, pCH35, pCH39, and pCH40 were prepared for nucleotide sequence analysis by creating a series of progressive deletions in the plasmids using the procedure of Barnes, Bevan, and Son (1983). Single-stranded DNA was isolated after superinfection of cultures of JM101 transformants with the helper phage IR1 (Dente, Cesareni, and Cortese, 1983) and sequenced using



the chain termination method (Sanger, Nicklen, and Coulson, 1977). In all cases, the sequence of complimentary strands was determined.

### Construction of pCG1223 and pCG2226

Chitinase promoter fragments were generated by *Bal31* digestion of the deletion subclone pCH35 $\Delta$ 311. The chitinase insert of this subclone has a 5' end located at -1653, and the insert of the undeleted plasmid pCH35 starts at -2004. Purified pCH35 $\Delta$ 311 plasmid DNA was first digested with *SphI* and the linearized DNA incubated with *Bal31* for a time sufficient to remove the 287 bp of coding sequence (Maniatis, Fritsch, and Sambrook, 1982). Following repair of the ends with Klenow, *BamHI* linkers were added. The promoter fragments were excised by *HindIII* digestion and cloned into the polylinker region of pEMBL9+ and their 3' endpoints were determined by nucleotide sequence analysis. The *HindIII*-*BamHI* fragments of samples pBD3223 and pBD3226 having 3' endpoints at +27 and +50 were cloned upstream of the  $\beta$ -glucuronidase coding region in the binary vectors pBI101.1 and pBI101.2, respectively (Clontech Laboratories, Inc., Palo Alto, CA).

### Construction of Deleted Chitinase Genes

The intact chitinase gene was inserted as the 4.7-kb *HindIII*-*EcoRI* fragment into the binary vectors pZS761 and pZS762 to yield pZC135 and pZC35, respectively. pZS761 and pZS762 were constructed by Zora Svab and Pal Maliga, Advanced Genetic Sciences, Oakland, CA. Deleted chitinase genes with 5' endpoints at -1057, -846, and -575 were obtained during generation of progressive deletions in pCH35 for nucleotide sequence analysis of the 5B gene. The *HindIII*-*EcoRI* fragments were cloned into pZS762 to give pZC13, pZC108, and pZC6, respectively. The modified genes of constructs pZC177 and pZC689 arose by *Bal31* digestion of the deletion subclone pCH35 $\Delta$ 6. The 5' ends of these shortened genes are found at -422 and -44, respectively. The -195 deletion of pZC12 was obtained by *Apal* digestion of pCH35 $\Delta$ 6 followed by attachment of *HindIII* linkers and cloning of the *HindIII*-*EcoRI* fragment in pZS762. In all cases, the 5' endpoints of the deleted chitinase genes were determined by nucleotide sequence analysis using the chain termination method.

### Transformation and Regeneration of Tobacco Plants

Constructs in binary DNA vectors were mobilized from *Escherichia coli* HB101 into *Agrobacterium tumefaciens* strain LBA 4404 by conjugation (Ruvkin and Ausubel, 1981) using *E. coli* HB101 harboring the mobilization plasmid pRK2013 (Figurski and Helinski, 1979). *Agrobacterium* cells containing the binary vectors were used to infect leaf discs of *Nicotiana tabacum* cv Xanthi essentially as described by Horsch et al. (1985). Kanamycin-resistant plants were grown in controlled environmental chambers maintained for a 12-hr, 24°C day and for a 12-hr, 20°C night cycle, with approximately 80% relative humidity, under mixed cool white fluorescent and incandescent lights.

### Ethylene Treatment of Plants

*Phaseolus vulgaris* cv Saxa were grown in growth chambers with a day/night photoperiod of 16 hr (22°C):8 hr (18°C) and used 7 days to 10 days after imbibition of dry seeds. Primary tobacco transformants were allowed to grow for 3 weeks to 5 weeks after transplanting into soil prior to ethylene treatment. Plants were then placed in airtight Lucite chambers and exposed to a continuous atmosphere of 50 ppm ethylene in air for 36 hours to 40 hours. After this time, samples of leaf tissue were harvested for analysis. The remainder of the tobacco transformants were allowed to recover and set seed.

### RNA Isolation and RNA Gel Blot Analysis

Total RNA was extracted from untreated and ethylene-treated plants, using guanidinium thiocyanate as a protein denaturant (Chirgwin et al., 1979) and further purified by centrifugation through a CsCl cushion (Glisin, Crkvenjakov, and Byus, 1974). Aliquots of RNA were denatured in glyoxal at 50°C and fractionated by electrophoresis on a 1% agarose gel. The RNA was blotted onto nitrocellulose filters and hybridized with nick-translated probes (specific activity 2 to 4  $\times 10^8$  cpm/ $\mu$ g) and the filters washed as described previously (Broglie, Gaynor, and Broglie, 1986). mRNA levels were quantitated by scanning autoradiographs on a model 300A computing densitometer (Molecular Dynamics, Sunnyvale, CA).

### $\beta$ -Glucuronidase Enzymatic Assays

Soluble protein was isolated from untreated and ethylene-treated plants essentially as described by Jefferson, Kavanagh, and Bevan (1987). Assays of GUS enzymatic activity were performed in 1 mL of extraction buffer [50 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, 10 mM mercaptoethanol] containing 1 mM 4-methylumbelliferyl glucuronide and 40  $\mu$ g of soluble tobacco protein. At 0 hr, 1 hr, 2 hr, and 3 hr, 200  $\mu$ L of the assay mixture was diluted into 0.1 M  $\text{Na}_2\text{CO}_3$  and the fluorescence at 455 nm determined on a Perkin-Elmer model LS-3B spectrofluorometer using an excitation wavelength of 365 nm.

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