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Regulation of a Chitinase Gene Promoter by Ethylene and Elicitors in Bean Protoplasts[1]

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ABSTRACT

Chitinase gene expression has been shown to be transcriptionally regulated by a number of inducers, including ethylene, elicitors, and pathogen attack. To investigate the mechanism(s) responsible for induction of chitinase gene expression in response to various stimuli, we have developed a transient gene expression system in bean (*Phaseolus vulgaris*) protoplasts that is responsive to ethylene and elicitor treatment. This system was used to study the expression of a chimeric gene composed of the 5′ flanking sequences of a bean endochitinase gene fused to the reporter gene β-glucuronidase linked to a 3′ fragment from nopaline synthase. Addition of 1-aminocyclopropane-1-carboxylic acid, the direct precursor of ethylene, or elicitors such as chitin oligosaccharides or cell wall fragments derived from *Colletotrichum lagenarium*, to transformed protoplasts resulted in a rapid and marked increase in the expression of the chimeric gene. The kinetics and dose response for these treatments were similar to those observed for the native gene *in vivo*. Analyses of 5′ deletion mutants in the protoplast system indicated that DNA sequences located between −305 and −236 are important for both ethylene and elicitor induction of the reporter gene.

Chitinase, a lytic enzyme found in most higher plants (1, 3), catalyzes the hydrolysis of chitin, a β-(1,4)-linked polymer of 2-acetamido-2-deoxy-β-D-glucose. Although no endogenous substrate for this enzyme has been found in plants, chitin is a major component of the cell walls of many fungi (31). For this reason, Abeles *et al.* (1) proposed that chitinase functions as a defense against chitin-containing pathogens. This hypothesis is supported by studies which indicate that chitinase levels are increased in response to pathogen attack (19, 20, 24, 25). *In vitro* studies have demonstrated further that plant chitinases are capable of hydrolyzing the cell walls of plant pathogenic fungi (30) and releasing elicitors of defense reactions (14). In addition, plants treated with the phytohormone ethylene (1, 3) or elicitors such as isolated fungal cell walls (7, 21, 22), endogenous plant cell walls (21), or chitin oligosaccharides (14, 23), have also been shown to contain increased levels of chitinase. A corresponding increase in chitinase mRNA levels has also been observed in these plants.

Recent studies indicate that the modulation of chitinase levels in plants involves the activation of gene transcription (15). In bean plants, chitinase is encoded by a multigene family consisting of at least three members (6). Of the three chitinase genes found in bean, the CH5B gene encodes an abundant mRNA that is known to be subject to induction by ethylene (5). We are interested in understanding the factors responsible for gene activation by ethylene and elicitors and the mechanism by which these signals are perceived by plant cells. Protoplasts have recently been shown to provide an excellent model system for such studies (9, 12, 13, 16, 29). Accordingly, we have developed a bean protoplast transient gene expression system that is responsive to ethylene and elicitor treatment. In this paper we report that ACC,[3] the direct precursor of ethylene (2), glycopeptide elicitors from *Colletotrichum lagenarium*, and a chitin heptamer cause a rapid and marked expression of a reporter gene under the control of a bean chitinase promoter. The induction kinetics and the dose-response for these treatments closely resemble those observed for the native gene *in vivo*. Analysis of chimeric gene constructs harboring 5′ deletions in the chitinase promoter fragment yields results that are qualitatively similar to those obtained when 5′ deleted chitinase genes are analyzed for ethylene-regulated expression in transgenic tobacco plants. The bean transient expression system can thus be used to investigate *cis*-acting regulatory sequences in the upstream region of the bean chitinase 5B gene that respond to ethylene or elicitors.

MATERIALS AND METHODS

Plasmid Constructions

The construction of the chimeric chitinase/gus A fusion gene has been previously described (5). The chimeric gene was cloned into the plasmid vector pUC18 and designated pGN2226. The 1.7 kb *Hin*dIII-*Bam*HI promoter fragment of pGN2226 served as the starting point in the construction of the chitinase promoter deletions. Digestion of this promoter fragment with *Taq*I resulted in a 1.65 kb fragment that served as the promoter fragment of pGTB26. The deletion fragments of constructs pGTB3, pGTB13, pGTB20, pGTB41, pGTB30, and pGTB17 were derived by *Bal*31 digestion of *Hin*dIII

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[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; kb, kilobase; GUS, β-glucuronidase.

linearized pGTB26. The promoter fragments of pGN6, pGN10, pGN18, and pGN21 were generated by polymerase chain reaction amplification of chitinase promoter sequences using pGTB30 as the template DNA. In each case, the 3′ end of the promoter fragment was defined by a 37 nucleotide oligomer encompassing positions +28 to +50 of the CH5B gene and containing a *Bam*HI site. The 5′ ends of the promoter fragments of pGN6, pGN10, pGN18, and pGN21 were situated at positions −305, −236, −173, and −107, respectively, and were obtained using synthetic oligonucleotides of 34 to 37 residues in length and bearing a *Hin*dIII site for cloning. Amplification reactions were performed using the GeneAmp Amplification Reagent Kit (Perkin Elmer Cetus, Norwalk, CT) with 40 pmol of primers and 0.03 fmol of target DNA. Samples were subject to 35 cycles of amplification. Amplified fragments were collected by ethanol precipitation and purified by electrophoresis on low melting agarose gels. All of the deleted chitinase promoter fragments were cloned in pUC12 as *Hin*dIII-*Bam*HI fragments upstream of a *Bam*HI-*Eco*RI fragment bearing the β-glucuronidase coding sequence linked to the termination signals of the nopaline synthase gene. In all cases, the 5′ end points of the deletion segments were determined by nucleotide sequence analysis using the dideoxy chain termination procedure (26).

Plant Material and Protoplast Isolation

Seeds of *Phaseolus vulgaris* L cv Saxa were purchased from La Main Verte (Toulouse, France). Plants were grown at 25°C with a day/night period of 16/8 h. Primary leaves of 20-d-old bean seedlings were used for isolation of protoplasts. Protoplasts were prepared by incubating leaf sections in Petri dishes containing 12 mL of 5 mM Mes buffer, pH 6.0, containing 0.35 M mannitol, 1 mM $CaCl_2$, 1.5% (w/v) driselase (Sigma), 0.3% pectinase (Worthington, medium I). In later experiments, higher levels of gene expression were obtained when an alternate digestion medium (medium II) was used for protoplast isolation. This medium consisted of 1 g/L cellualse R10 Onozuka, 200 mg/L macerozyme Onozuka (Yakult Honsha, Nishinomiya, Japan) and 500 mg/L pectinolyase Y23 (Seishin Pharmaceutical Ind.) in modified Murashige and Skoog medium (T0 medium, ref. 8). In most experiments, AVG was added at the concentrations indicated in the text. The leaf sections were incubated in the digestion medium for either 3 h at 28°C in darkness (medium I) or for 15 h at 22°C in darkness (medium II). Protoplasts were separated from the cellular debris by sieving through an 85 μm nylon mesh followed by centrifugation at 20*g* for 5 min onto a 1 mL cushion of 19% (w/v) sucrose. Intact protoplasts, which floated on the sucrose cushion, were removed, washed once with 5 mM Mes buffer, pH 6.0, containing 0.4 M mannitol and 1 mM $CaCl_2$, and counted.

Transient Expression Assays

In most experiments, protoplast transformation was performed by electroporation. Prior to electroporation, protoplasts were washed in electroporation medium (50 mM acetate buffer, pH 4.0, 100 mM KCl, and 0.5 M mannitol) and adjusted to the desired density (2–2.5 × 10^6 protoplasts/mL). A 250 μL sample of washed protoplasts was mixed with 50 μg of plasmid DNA and transferred to the electroporation chamber. Electroporation was carried out by discharging a 500 μF capacitor charged at 50 V. Two electric pulses were delivered, separated by 30 s. The protoplasts were then incubated in electroporation buffer for 10 min. In other experiments, transformation was achieved by adding 160 μL of a PEG solution (40% PEG, 0.4 M mannitol, 30 mM $MgCl_2$, 0.1% Mes, pH 5.8) to a mixture of protoplast (320 μL samples) and plasmid DNA (50 μg per assay in 10 mM Tris-HCl, pH 8.0). Following transformation, the protoplasts were collected by centrifugation and resuspended in 500 μL of Gamborg's B5 medium supplemented with 0.3 M mannitol, 0.1 M glucose, 0.1% casein hydrolysate, 1 mg/mL $CaCl_2$, and 1 mg/L 2,4-D with or without the addition of 100 μM ACC, 100 μg/mL purified fungal elicitor from *Colletotrichum lagenarium* (28), or 25 μM chitin heptamer (4). Protoplasts were incubated at 22°C for 0.5 to 48 h. After incubation, the protoplasts were collected and lysed by the addition of either 500 μL GUS buffer or 50 μL of 10 × GUS buffer (17). Cellular debris was removed from the extract by centrifugation, and the supernatant assayed for β-glucuronidase activity using a spectrofluorometric assay (17). Protein was determined using the Bradford dye reagent.

Elicitor and ACC Treatment of Plants

Young bean seedlings (7-d-old) were excised at the stem base and incubated for various times in 10 mM phosphate buffer, pH 6.0, under growth conditions. Elicitor-treated samples contained either fungal elicitors isolated from *C. lagenarium* (ethanol-soluble fraction, as described in ref. 28) or a chitin oligosaccharide (oligomer 7, as described in ref. 4) at final concentrations of 50 μM. Ethylene measurements were performed using a Shimadzu GC-8A gas chromatograph.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from bean seedlings using a scaled-down version of the procedure described by Roby and Esquerre-Tugaye (25). RNA was purified further by precipitation with lithium chloride. Samples were denatured in glyoxal at 50°C, fractionated by electrophoresis on a 1% agarose gel, and blotted onto Hybond N membrane (Amersham). The RNA was fixed to the filter by UV irradiation followed by heating for 1 h at 65°C according to the manufacturer's specifications. RNA blots were hybridized with nick-translated insert (specific activity 2–4 × 10^6 cpm/μg) from chitinase cDNA clone, pCH18 (6). Filters were washed once with 2 × SSC (0.75 M NaCl, 0.03 M sodium citrate, pH 7.0) containing 0.1% SDS for 15 min at room temperature, once with 1 × SSC under the same conditions, and twice with 0.1 × SSC containing 0.1% SDS for 30 min at 55°C.

RESULTS

Ethylene and Elicitor Induction of Endogenous Chitinase Genes

Chitinase mRNA synthesis has been shown to be stimulated in bean cell suspensions by treatment with fungal cell wall

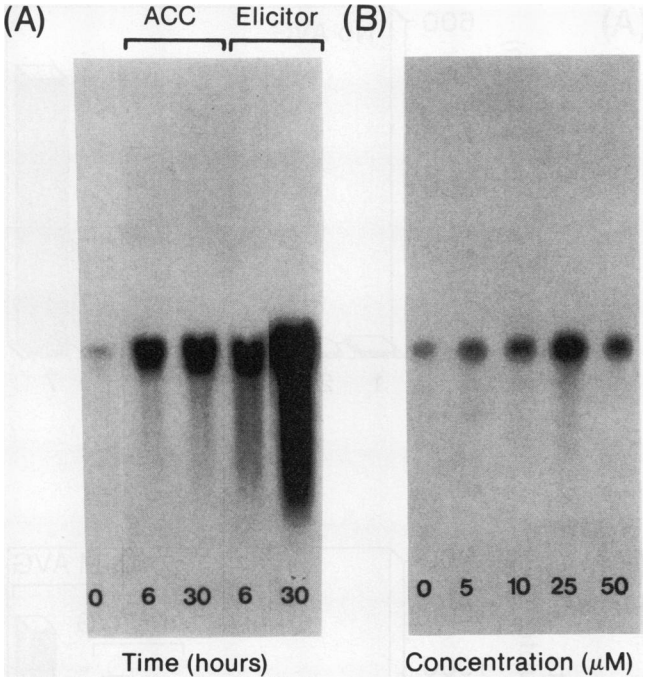

Figure 1. Northern blot analyses of chitinase mRNA induction by ACC and elicitor treatment. *A*, Time course of chitinase mRNA accumulation in bean seedlings following treatment with either 50 μM ACC or 400 μg glucose equivalents/mL of an elicitor purified from *C. lagenarium*. *B*, Effect of varying concentrations of chitin oligosaccharide (oligomer 7) on chitinase mRNA accumulation in bean seedlings. Plants were treated for 16 h; lanes contained 10 μg total RNA. Blots were hybridized with nick-translated insert fragment from chitinase cDNA clone pCH18.

elicitors (15). Similarly, we have shown that chitinase mRNA levels are increased in bean leaves following exposure to exogenous ethylene (6). To determine the effect of elicitor treatment on chitinase mRNA levels in intact leaves, young bean seedlings were excised at the base of the stem and incubated with either fungal cell wall elicitors, a purified chitin oligosaccharide, or the ethylene precursor, ACC. As shown in Figure 1, only low levels of chitinase mRNA are detected prior to treatment or when plants are treated with buffer alone. In the presence of 50 μM ACC, chitinase mRNA levels are found to increase within 6 h of treatment and continue to rise for at least 30 h. Similar results are obtained with 400 μg glucose equivalents/mL of a crude elicitor preparation from *C. lagenarium*. When a purified preparation of chitin oligosaccharide is used, concentrations as low as 5 μM are sufficient to induce chitinase gene expression. The optimum concentration for chitinase mRNA induction using the purified chitin oligosaccharide is 25 μM.

Ethylene- and Elicitor-Induced Transient Expression of a Chimeric Chitinase/*gus* A Gene in Bean Protoplasts

To study the mechanism of chitinase gene activation by ethylene and elicitors, a transient expression system has been developed using protoplasts isolated from young bean leaves. Protoplasts are excellent tools to study transcriptional regulation quickly and efficiently, avoiding the potential complications of position effect and variable gene copy numbers often associated with stable transformation. However, a prerequisite for the use of protoplasts is their ability to perceive and respond to exogenous signals such as those provided by elicitors and ACC. To test this capability, freshly isolated protoplasts were treated for 20 h with increasing amounts of ACC or fungal elicitor and assayed for production of ethylene. In the absence of added inducers, bean protoplasts produce low levels of ethylene, probably as a result of wounding associated with their isolation. However, as shown in Figure 2B, the level of ethylene produced by the protoplast suspen-

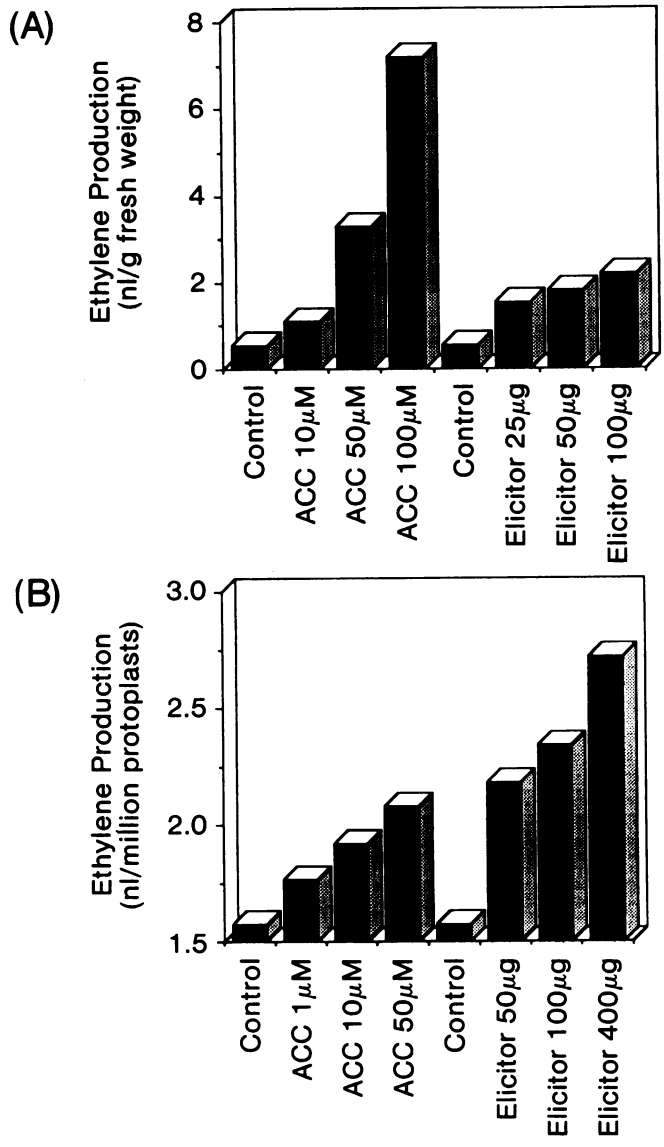

Figure 2. Ethylene production following elicitor and ACC treatment of bean seedlings and protoplasts. *A*, Ethylene production in bean seedlings treated with increasing amounts of ACC or *C. lagenarium* elicitor. *B*, Ethylene production by protoplasts following treatment with increasing concentrations of either ACC or fungal elicitor from *C. lagenarium*. Ethylene levels were measured after 20 h of treatment.

sion increases in the presence of ACC or elicitor; the amount of ethylene produced is proportional to the concentration of ACC or elicitor added. These results may be compared to those obtained when bean seedlings are treated with ACC or elicitor (Figure 2A). Although the data are qualitatively similar in both cases, the increase in ethylene production associated with elicitor treatment is greater in protoplasts than in intact leaves. This may reflect problems associated with the uptake of elicitor into intact plants. Such complicating factors either do not exist or are minor in the case of protoplasts. Thus, cultured bean protoplasts are able to sense and respond to appropriate signals for ethylene production. The fact that this response is qualitatively analogous to that found in intact plants makes it a suitable system for the rapid analysis of chitinase promoter function.

To investigate the response of the chitinase promoter to ethylene and elicitor treatment, a chimeric gene was constructed and introduced into bean protoplasts by electroporation. The chimeric gene is composed of a translational fusion between 1.7 kb of 5′ flanking sequences from the chitinase 5B gene and the coding region of the reporter gene β-glucuronidase, linked to the 3′ untranslated region of nopaline synthase (Fig. 3). The chitinase/*gus* A fusion gene has been incorporated into the binary vector pBI101.2 and the resultant binary plasmid pCG2226 was introduced into tobacco plants by *Agrobacterium tumefaciens*-mediated transformation. An analysis of transgenic plants harboring this construct has revealed that the *gus* A gene, under the control of the chitinase 5B promoter, is induced when plants are treated with the phytohormone ethylene. Thus, the 5′ flanking region of the 5B gene alone seems to be sufficient to confer ethylene-regulated expression to the reporter gene (5).

The bean transient expression system was initially characterized and optimized by studying the response of the chimeric *gus* A gene to induction by the ethylene precursor ACC. pGN2226 was electroporated into bean protoplasts as described in "Materials and Methods." Following transformation, the protoplasts were gently washed and exchanged into medium containing 100 μM ACC. After incubation for 48 h, the protoplasts were lysed and assayed for expression of β-glucuronidase enzyme activity. Figure 4A shows that protoplasts that are electroporated in the absence of added DNA or protoplasts that are not electroporated but which contain added DNA, exhibit low or undetectable levels of endogenous β-glucuronidase enzyme activity. When protoplasts are electroporated with pGN2226 and then incubated in the presence of ACC, GUS activity is significantly increased. As a control

Figure 3. Restriction map of the chimeric β-glucuronidase gene pGN2226. This gene consists of a translational fusion between a 1.7 kb fragment of the bean chitinase CH5B gene and the coding region of β-glucuronidase linked to the nopaline synthase polyadenylation signal.

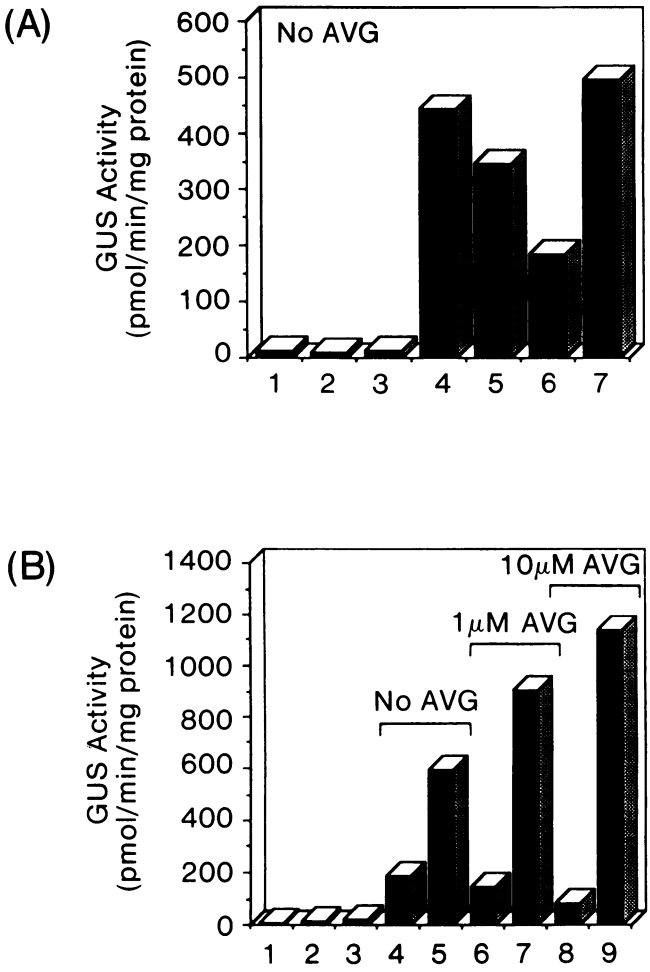

Figure 4. Effect of ACC treatment on CaMV 35S and chitinase promoter activity in electroporated bean protoplasts. *A*, β-Glucuronidase enzyme activity in protoplasts electroporated with control plasmid pBI221 (lanes 4 and 5) of pGN2226 (lanes 6 and 7), in the presence (lanes 5 and 7) or absence (lanes 4 and 6) of 100 μM ACC. Lane 1, no treatment; lane 2, electroporation without DNA; lane 3, DNA without electroporation. Samples were assayed after 48 h incubation. *B*, Effect of AVG on β-glucuronidase activity in electroporated protoplasts. In lanes 4 and 5 protoplasts were isolated in the absence of the ethylene biosynthesis inhibitor AVG; in lanes 6 and 7 and 8 and 9, protoplasts were isolated in the presence of 1 and 10 μM AVG, respectively. Assays were performed after 48 h of incubation.

in this experiment, β-glucuronidase activity was measured in protoplasts electroporated with the plasmid pBI221. In this case, expression of the *gus* A gene under control of the cauliflower mosaic virus 35S promoter is high in untreated protoplasts and undergoes a slight decrease (22%) in the presence of ACC.

In the previous experiment, protoplasts that were electroporated with pGN2226 but not treated with ACC were also found to express low but significant levels of GUS activity. This may be due to ethylene production and/or release of endogenous elicitors during protoplast isolation (11, 12). In order to decrease this background stimulation, an inhibitor

of ethylene biosynthesis, AVG, was added to the protoplast isolation medium. As shown in Figure 4B, concentrations of AVG ranging from 1 to 10 μM were found to reduce β-glucuronidase expression in untreated protoplasts. GUS enzyme activity remains inducible by ACC, with the enzyme levels being somewhat greater than in protoplasts isolated in the absence of added AVG. This apparent stimulatory effect may be due to improved protoplast viability as a result of inhibition of ethylene biosynthesis. Consistent with this suggestion, expression of GUS activity from pBI221 is found to increase when protoplasts are prepared in the presence of 1 μM AVG (data not shown). Because it served to lower background levels of GUS activity present in the absence of added inducer and also proved to enhance the level of enzyme induction, AVG was routinely included in the digestion medium employed to generate the bean protoplasts.

In addition to the ethylene precursor ACC, fungal elicitors also stimulate expression of the chimeric *gus* A gene in the bean transient expression system. Bean protoplasts, isolated in the presence of 10 μM AVG, were electroporated with pGN2226 and then incubated for 24 h with increasing concentrations of chitin oligosaccharide. As shown in Figure 5, the level of β-glucuronidase enzyme activity, driven by the chitinase 5B promoter, is proportional to the concentration of added elicitor. Induction is evident at concentrations as low as 1 μM, with maximal expression being achieved at 25 μM chitin heptamer. A purified fungal elicitor from *C. lagenarium* also induces expression of the chimeric gene in bean protoplasts at comparable levels (data not shown).

To characterize better the response of the transient expression system to the two inducers, the kinetics of induction of GUS enzyme activity was determined for ACC (100 μM) and the chitin heptamer (25 μM). Figure 6 shows that a low but

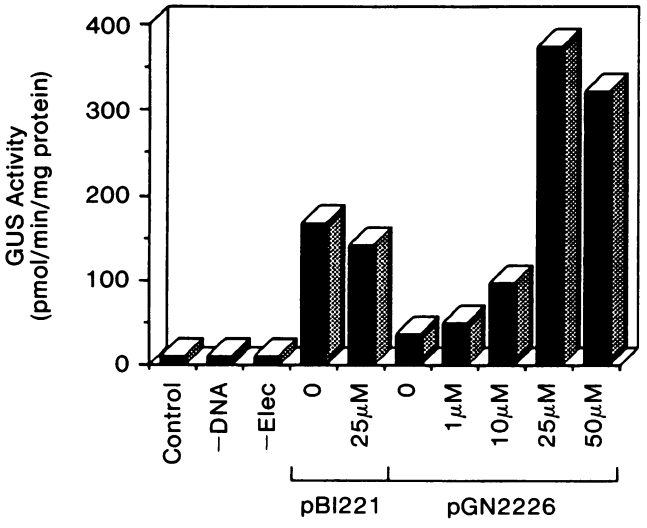

Figure 5. Effect of elicitor treatment on CaMV 35S and chitinase promoter activity in electroporated bean protoplasts. Control, untreated protoplasts; −DNA, protoplasts electroporated without added DNA; −Elec, protoplasts with DNA but without electroporation; pBI221, control plasmid; pGN2226, chitinase/*gus* A fusion. Elicitor concentrations are indicated on the figure. Samples were assayed for GUS activity after 48 h of treatment.

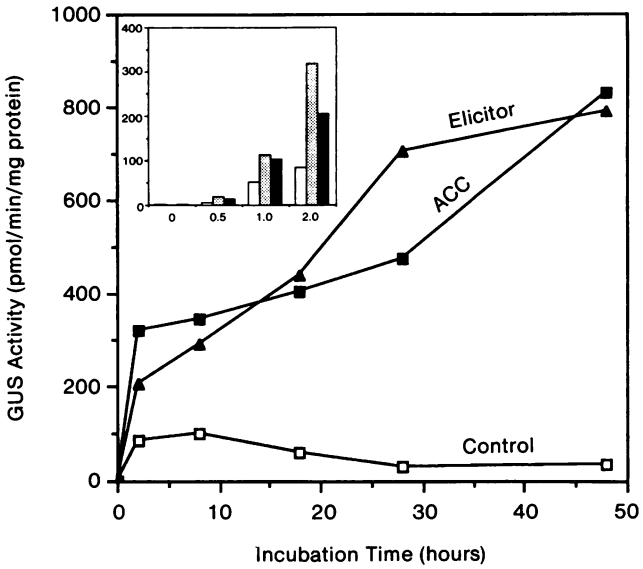

Figure 6. Time course of elicitor and ACC induction of β-glucuronidase enzyme activity following electroporation of bean protoplasts with the chimeric chitinase/*gus* A gene. (—□—) untreated controls; (—■—) 100 μM ACC; (—▲—) 25 μM chitin heptamer.

detectable induction of GUS activity is observed for both treatments after incubation times as short as 30 min. The level of GUS activity continues to rise with time, reaching an approximately 25-fold stimulation over control values after 48 h treatment with either ACC or chitin heptamer.

Analysis of 5′ Deletions in the Promoter Fragment of pGN2226

Previous studies of deleted chitinase 5B genes in transgenic tobacco indicate that sequences downstream from −575 are required for efficient, regulated expression by the phytohormone, ethylene (5). To delineate further the sequences necessary for induction by ethylene and elicitor, a series of 5′ deletions in the promoter fragment of pGN2226 was constructed and analyzed in bean protoplasts. All of the constructs contain identical translational fusions to the coding region of β-glucuronidase with the polyadenylation signals being provided by the nopaline synthase 3′ end fragment. Bean protoplasts were electroporated with equivalent amounts of the chimeric genes contained in pUC12 and then treated with either 100 μM ACC or 25 μM chitin heptamer. The results of the GUS enzymatic assays performed on the protoplast extracts are shown in Figure 7. Deletion of the promoter fragment to −453 decreases by 38% the level of GUS enzyme activity found in extracts of ACC-treated protoplasts, whereas the level of GUS enzyme activity following elicitor treatment decreases by 12%. Further shortening of the promoter segment to −305 results in a 62% decrease in GUS enzyme activity relative to the pGN2226 control. When DNA sequences between −305 and −236 are removed, GUS activity declines to the control level displayed in extracts of untreated protoplasts. Treatment of electroporated protoplasts with fungal elicitor yields qualitatively similar results. GUS enzyme

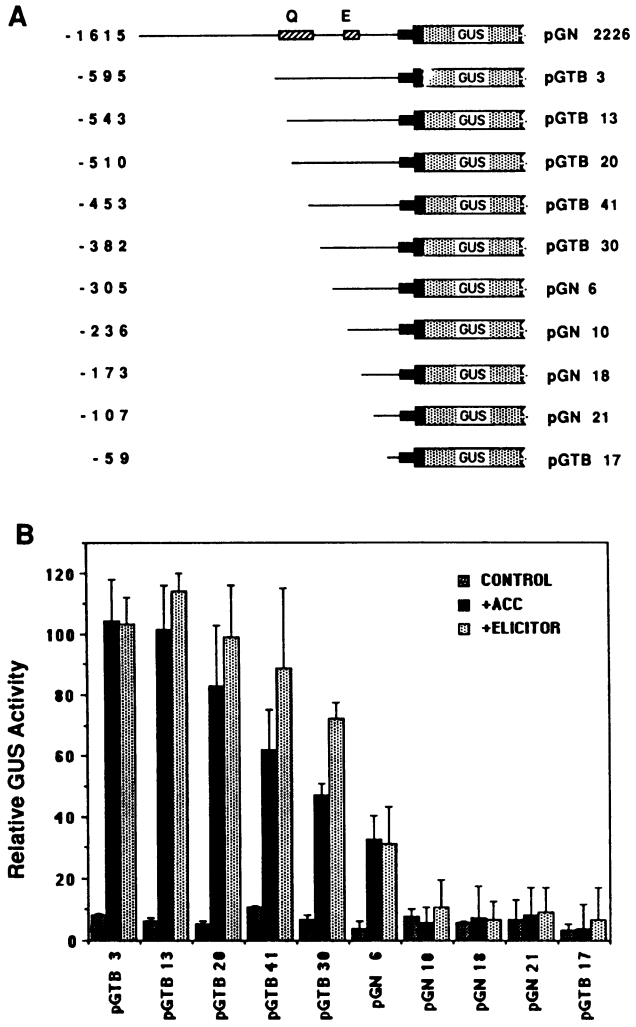

Figure 7. Identification of ethylene- and elicitor-responsive *cis*-elements by transient expression. *A*, 5′ deletions of the CH5B gene promoter. The deletion end points are indicated on the left and are numbered from the transcriptional start site. Q and E refer to regions of the CH5B gene promoter involved in high level and ethylene-regulated expression, respectively. *B*, Results of GUS enzymatic assays of various deletions following elicitor- and ACC-treatment of protoplasts electroporated with individual deleted promoter constructs. The data shown represent the mean of three separate experiments.

activity decreases as upstream regions of the chitinase promoter are deleted to −453. A significant decrease (62%) in GUS enzyme levels is observed as the 5′ end is moved from −510 to −305. Finally, all inducible GUS enzyme activity is lost as the segment between −305 and −236 is removed.

DISCUSSION

In this paper, evidence is presented for ethylene and elicitor regulation of a chitinase gene promoter in transformed bean protoplasts. As with other transient expression systems designed to study the regulation of plant defense genes (9, 12), bean leaf protoplasts are found to perceive and respond to external stimuli. This is despite a background stimulation in the absence of added inducers that is presumably due to production of ethylene and/or release of endogenous elicitors during protoplast preparation and isolation (10, 11, 18). Our data indicate that this background may be significantly reduced by inclusion of the ethylene biosynthesis inhibitor AVG in the protoplast digestion medium. Moreover, the presence of this inhibitor appears to give rise to enhanced levels of GUS activity in protoplasts electroporated with pGN2226 and treated with ACC. This latter effect may result from increased protoplast viability that accompanies inhibition of ethylene formation.

Expression of the chimeric gene of pGN2226 in bean protoplasts is regulated by ethylene and elicitors in a manner similar to that of the endogenous bean chitinase gene *in vivo*. The protoplast system is rapidly activated by both ACC and chitin heptamer within 30 min. The fast response time suggests that only a few steps intervene between signal recognition and gene activation. Because bean protoplasts activate transcription of the chimeric chitinase/*gus* A gene, an intact cell wall is not required for recognition or transmission of the signal(s) for induction by ACC and elicitors. Presumably, these stimuli act by binding to a component(s) of the plant cell membrane. A similar conclusion has been noted by Dangl *et al.* (9) in their analysis of the response of parsley protoplasts to UV light and treatment with fungal elicitor.

The development of a bean transient expression system responsive to ethylene and fungal elicitors enables a rapid evaluation of chitinase promoter function. Analysis of deleted chitinase genes in transgenic tobacco plants has indicated that DNA sequences located downstream of −595 are involved in ethylene induction of chitinase gene expression. This study suggested that the promoter of the chitinase 5B gene is, at least in part, composed of a quantitative transcription element located between −595 and −422 that influences absolute levels of transcription and an ethylene responsive element that resides between positions −422 and −44 (5). These results, obtained in the heterologous tobacco system, are confirmed and extended in the homologous bean protoplast assay system. Evaluation of chitinase promoter deletions fused to the reporter gene, β-glucuronidase, shows the presence of a quantitative element between −510 and −305, although the exact borders of this element are less well defined by transient expression assays compared with analysis of stable transformants (K. Broglie, R. Broglie, unpublished results). This may be due to the fact that nonchromatin-associated DNA is being transcribed in the transient system. The ethylene responsive element appears to be located further downstream between −305 and −236. Assay of the chimeric gene deletions in the presence of a chitin oligomer shows that the functional profile for this inducer is qualitatively similar to that for the phytohormone ethylene. Moreover, the time courses for induction by the two compounds are also strikingly similar. These results may directly reflect the known stimulation of ethylene formation by fungal elicitors (7, 21, 22, 27). Whether an ethylene-independent contribution to chitinase induction by elicitor is present but masked cannot be ruled out in this study. A finer mapping of the chitinase promoter using the bean transient expression system will allow a more precise definition of the ethylene and elicitor responsive elements. These

experiments, combined with studies of the binding of *trans*-acting factors, may provide some insights concerning the mode of regulation of chitinase gene expression by ethylene and fungal elicitors.

ACKNOWLEDGMENTS

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