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Developmental and Pathogen-Induced Activation of the Arabidopsis Acidic Chitinase Promoter

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Expression of the Arabidopsis acidic chitinase promoter was investigated during plant development and in response to inoculation with fungal pathogens. A chimeric gene composed of 1129 bp of 5′ upstream sequence from the acidic chitinase gene was fused to the β-glucuronidase (GUS) coding region and used to transform Arabidopsis and tomato. Promoter activity was monitored by histochemical and quantitative assays of GUS activity. In healthy transgenic plants, the acidic chitinase promoter activity was restricted to roots, leaf vascular tissue, hydathodes, guard cells, and anthers, whereas GUS expression was induced in mesophyll cells surrounding lesions caused by *Rhizoctonia solani* infection of transgenic Arabidopsis. In transgenic tomato plants, GUS expression was induced around necrotic lesions caused by *Alternaria solani* and *Phytophthora infestans*. Expression of the acidic chitinase promoter-GUS transgene was weakly induced by infiltrating leaves with salicylic acid. Analysis of a series of 5′ deletions of the acidic chitinase promoter in Arabidopsis indicated that the proximal 192 bp from the transcription initiation site was sufficient to establish both the constitutive and induced pattern of expression. Elements further upstream were involved in quantitative expression of the gene. The location of a negative regulatory element was indicated between −384 and −590 and positive regulatory elements between −1129 and −590.

INTRODUCTION

Chitinases have been purified from a wide variety of higher plant species. These enzymes are members of a group of proteins known as pathogenesis-related (PR) proteins that increase in response to pathogen infection and certain abiotic stresses (Boller, 1988). Based on their amino acid sequences, there are at least three classes of plant chitinases (Shinshi et al., 1990). The class I chitinases are basic proteins that contain a highly conserved cysteine and glycine-rich N-terminal domain with putative chitin-binding properties. Expression of the class I chitinases from bean and tobacco is induced by ethylene (Broglie et al., 1986; Memelink et al., 1990), and the mature enzyme appears to be localized primarily in the plant cell vacuole (Boller and Vogeli, 1984; Keefe et al., 1990). The class II enzymes have a high degree of sequence similarity to class I enzymes but lack the chitin binding domain and are acidic proteins (Shinshi et al., 1990). Class II chitinases from tobacco are found in intercellular washing fluids (Parent et al., 1985) and, therefore, are probably associated with the cell wall or excreted into the apoplasm. Class III chitinases lack sequence similarity to class I or II enzymes and may be basic or acidic proteins. Enzymes in class III include the acidic extracellular cucumber chitinase (Metraux et al., 1988, 1989) and the Hevea latex chitinase (Rozeboom et al., 1990).

Chitinases may function in defending plants from fungal and bacterial pathogens. Chitinases accumulate after pathogen attack (Boller, 1988) and purified plant chitinases have antifungal (Schlumbaum et al., 1986; Broekaert et al., 1988; Roberts and Selitrennikoff, 1988) and lysozymal (Trudel et al., 1989; Martin, 1991) activity in vitro. Chitin, a polymer of *N*-acetyl-D-glucosamine, is not found in plants but is a major component of the cell walls of many fungi (Bartnicki-Garcia, 1968). Chitinases may be involved in direct inhibition of fungal growth and/or in the release of defense-related elicitors from fungal cell walls. However, it is possible that an unknown chitinase substrate exists in plants and that these enzymes have an as yet unrecognized role in plant growth and development.

The class I and II chitinases are expressed in an organ-specific and age-dependent manner in uninfected plants. Class I chitinases are highly expressed in roots and basal leaves of tobacco (Shinshi et al., 1987; Neale et al., 1990), whereas the class II chitinases are expressed at much lower levels in these organs (Memelink et al., 1990). Moderate levels of both class I and II chitinases occur in tobacco flowers (Lotan et al., 1989; Memelink et al., 1990; Neale et al., 1990).

Expression of the cucumber class III chitinase is induced by pathogen invasion and the enzyme accumulates in the extracellular space of infected and healthy leaves (Metraux et al., 1988). However, the tissue-specific pattern of expression of class III chitinase in either healthy or diseased plants has not been previously explored. We reported earlier the cloning of the single-copy class III chitinase gene encoding an acidic chitinase from Arabidopsis

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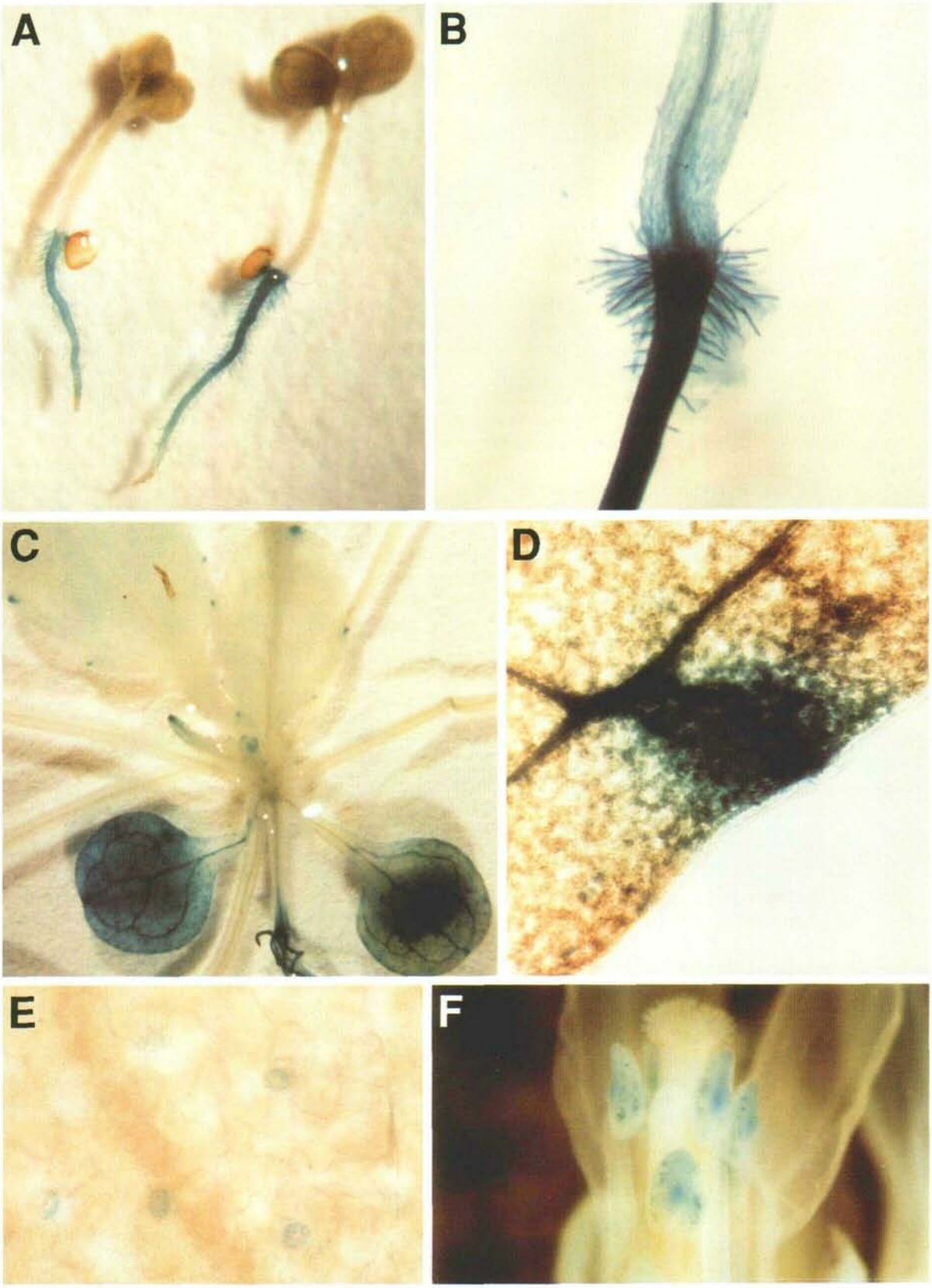

Figure 1. Histochemical Localization of GUS Expression in Transgenic Arabidopsis Plants Containing pMON8896.

(Samac et al., 1990). To study the developmental and inducible pattern of expression of this gene, we generated transgenic Arabidopsis plants expressing β-glucuronidase (GUS) under the control of the acidic chitinase promoter. Here we demonstrate that the chimeric gene is expressed constitutively in certain cell types of healthy plants and is induced in other cell types by fungal infection. Promoter deletion experiments identified regions in the promoter involved in the quantitative level of expression and organ specificity as well as induction by fungal infection.

RESULTS

Histochemical Analysis of Expression of the Acidic Chitinase Promoter-GUS Gene Fusion

A chimeric gene containing a 1129-bp fragment of the 5′ upstream sequence from the Arabidopsis acidic chitinase gene (Samac et al., 1990) in transcriptional fusion with the GUS gene and nopaline synthase poly(A) termination region was constructed (pMON8896) and introduced into Arabidopsis ecotype RLD by Agrobacterium-mediated transformation (Valvekens et al., 1988). Control plants containing a promoterless GUS gene construct and plants containing GUS driven by the cauliflower mosaic virus (CaMV) 35S promoter (pMON9749) were also generated. Seed from regenerated Arabidopsis plants were sown on medium containing 50 mg/L kanamycin (Valvekens et al., 1988), and kanamycin-resistant plants were stained for GUS activity.

Figure 1 shows the histochemical GUS activity in representative plants containing pMON8896 at different developmental stages. Ten independent transgenic lines were assayed and all had the same overall pattern of expression. Very rapid and strong GUS staining was found in roots (Figure 1A). In seedlings, all root cells appeared to have GUS activity, except the root tips of some plants, with cortical tissue staining more rapidly than vascular tissue. At the root crown, there was a sharp drop in GUS staining in all cells except the vascular tissue (Figure 1B). In roots from older plants, GUS staining was less intense and more limited to vascular tissue (data not shown). In leaves, strong GUS staining was seen in vascular tissue and, notably, in the hydathodes at the ends of veins along the leaf margins (Figures 1C and 1D). Close inspection also revealed GUS expression in the guard cells of open stomates (Figure 1E). GUS staining was also strong in senescent tissues, such as cotyledons of 3-week-old plants (Figure 1C). In the flowers, GUS staining was found in anthers (Figure 1F). No GUS activity was observed in control plants containing the promoterless GUS construct (data not shown). In plants containing pMON9749 (35S-GUS), all tissues stained uniformly blue except for a short length in the middle region of the hypocotyl which lacked GUS activity (data not shown).

To test the expression of the acidic chitinase promoter in a heterologous system, we generated five transgenic tomato lines containing pMON8896. GUS staining in tomato and Arabidopsis was similar (data not shown). Roots of tomato plants had very strong GUS staining and GUS was also expressed in leaf vascular tissue and hydathodes.

We have examined the expression of the acidic chitinase gene by RNA gel blotting experiments. In earlier experiments (Samac et al., 1990), we did not detect acidic chitinase mRNA in healthy plants. In subsequent experiments using higher levels of total RNA (50 μg per lane), faint signals were detected in lanes containing RNA extracted from roots of Arabidopsis ecotypes Columbia and RLD (data not shown). Histochemical analysis of GUS activity is a more sensitive assay for promoter activity than RNA analysis. The sensitivity of the assay appears to be due to greater stability of the GUS mRNA relative to the acidic chitinase message. RNA gel blots with RNA extracted from roots of Arabidopsis plants containing pMON8896 and then probed with the GUS coding sequence revealed substantial GUS mRNA levels (data not shown). In addition, the level of GUS activity observed may also be due to the accumulation of GUS, reportedly a very stable protein (Jefferson et al., 1987).

Effect of 5′ Deletions on Organ-Specific Expression

To establish the location of promoter elements required for gene expression, a number of 5′ end point deletions in the putative promoter were generated by the polymerase chain reaction (PCR) technique (see Methods). Figure 2A shows a diagram of the promoter deletion constructs and the end points of the fragments used in this analysis relative to the transcription initiation site.

Histochemical staining was carried out on transgenic Arabidopsis plants containing the 5′ deletion constructs. The intensity of staining decreased, and the rate of staining slowed significantly, in plants containing pMON8896Δ590 compared to plants containing the full-length 1129-bp

Figure 1. (continued).

(A) GUS staining in young seedlings 7 days after planting.
(B) Root crown of 2-week-old seedling.
(C) GUS staining in leaf vascular tissue, hydathodes, and senescent cotyledons of 3-week-old plant.
(D) Hydathode on leaf margin.
(E) Expression in guard cells of open stomates.
(F) Expression in inflorescence.

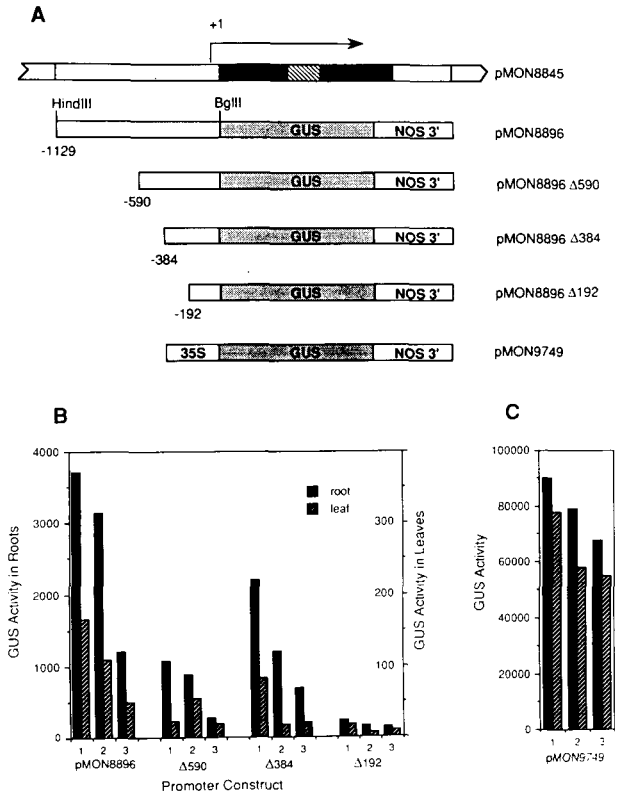

Figure 2. Structure of 5′ Promoter Deletion Constructs and Effect of Promoter Deletions on Level of GUS Activity in Roots and Leaves of Transgenic Arabidopsis Plants.

(A) Schematic representation of the acidic chitinase promoter-GUS constructs. Promoter fragments were isolated as described in Methods from pMON8845 containing the intact acidic chitinase genomic clone. Promoter length is indicated with respect to the transcription start site. In pMON8845, the black area indicates exons and the hatched area indicates the intron.

(B) GUS activity (picomoles of 4-methylumbelliferone per minute per milligram protein) was measured in roots and leaves of R_1 plants containing pMON8896 or the promoter deletion constructs illustrated in **(A)**. From each line, the roots and leaves of 10 2-week-old seedlings were harvested, pooled, and assayed for GUS activity. Four to seven lines of plants containing a single insertion of the promoter-GUS construct were assayed. Illustrated are the plant lines with the highest (1), average (2), and lowest (3) GUS activities.

(C) GUS activity in roots and leaves of plants from three representative lines containing pMON9749. Activity was measured as in **(B)**.

promoter construct (pMON8896). Whereas the plants containing pMON8896 required only 1 to 2 hr to develop full color, plants containing the promoter deletion constructs required 12 to 18 hr to develop full color. Interestingly, GUS activity appeared greater in plants containing pMON8896Δ384 than pMON8896Δ590, although activity was not as high as in plants containing pMON8896. Plants containing the 192-bp promoter, pMON8896Δ192, had the lowest GUS activity, but light-blue staining was still observed throughout the root, in leaf vascular tissue, hydathodes, and guard cells.

Figure 2B shows the levels of GUS enzymatic activity extracted from roots and leaves of 14-day-old plants containing the 1129-bp promoter or promoter deletion-GUS constructs. Each bar represents the GUS activity in roots or leaves from 10 pooled GUS positive R_1 plants derived from a single primary transformant (R_0). A range in expression levels for each construct was found, as has also been observed by others (Van de Rhee et al., 1990). Overall, these data correlate with the intensity of GUS expression observed by histochemical staining. In roots, deleting the promoter from −1129 to −590 decreased the average GUS activity to 33% of the level of activity in plants with the undeleted promoter. Further deletion of the promoter to −384 decreased activity by an average of 51% and deleting to −192 decreased GUS activity to 7% of the activity in plants with the 1129-bp promoter. In leaf extracts, GUS activity was relatively low in the plants containing pMON8896 and decreased in a step-wise manner when the promoter was progressively shortened. Compared to plants containing pMON8896, the averaged GUS activity in leaf extracts decreased 67% in plants containing pMON8896Δ590, 34% in plants containing pMON8896Δ384, and 25% in plants containing pMON8896Δ192. Five lines containing the promoterless GUS construct were assayed for GUS activity and four lines containing pMON9749 (35S-GUS). No GUS activity was detected in control plants containing the promoterless GUS construct. All the plant lines containing pMON9749 had similar levels of GUS activity (Figure 2C). Comparisons of the mean GUS activity in roots of plants containing pMON9749 and pMON8896 revealed that the level of GUS expression driven by the CaMV 35S promoter was, on average, 27-fold greater than the expression driven by the acidic chitinase promoter.

Promoter Induction by Fungal Pathogens

Relatively few known pathogens cause disease in Arabidopsis. Recently, an isolate of *Rhizoctonia solani* was identified which infects Arabidopsis (Koch and Slusarenko, 1990). Transformed Arabidopsis plants containing the 1129-bp promoter or the promoter deletion constructs were inoculated with *R. solani* to test whether expression of the acidic chitinase promoter is induced by fungal

infection. Plants were inoculated by placing 1 cm^2 agar plugs of mycelium on the soil near 4-week-old plants. Three days after inoculation, a web of hyphae had grown from the plugs onto leaves. Infected areas first yellowed, then became necrotic and rotted quickly. All inoculated plants became completely diseased and were dead 7 days after inoculation.

GUS staining of infected leaves was carried out on leaves in the relatively early stages of disease development. Before necrotic lesion development, GUS activity was observed in the mesophyll cells underlying infection cushions (data not shown). Figure 3 illustrates that, after lesion development, strong GUS activity appeared in the mesophyll bordering diseased tissue. GUS staining in the remaining healthy tissue was unchanged. Plants containing pMON8896 and promoter deletion constructs responded similarly to infection by *R. solani*. However, the intensity of GUS staining was lower in plants containing the promoter deletion constructs than in plants containing pMON8896.

Tomato leaves varied in susceptibility to *Alternaria solani*, depending on the age of the leaf. In mature fruiting plants, older leaves from the base of the plant were highly susceptible, whereas the younger leaves from the top of the plant were quite resistant. Both young and old tomato leaves from R_0 plants containing pMON8896 were inoculated with *A. solani* in a detached-leaf assay. Three days after inoculation, small necrotic lesions were observed on young and old leaves. Six days after inoculation, lesions on older leaves had greatly enlarged, with leaf yellowing occurring around lesions. In young leaves, lesions remained small and localized with no leaf yellowing. Strong GUS activity occurred around lesions on young leaves both 3 and 6 days after inoculation (Figure 3B). Only very weak GUS activity was observed in older leaves after infection (Figure 3C).

Phytophthora infestans is also a foliar leaf-spotting pathogen of tomato but, unlike *A. solani*, hyphal cell walls contain only minor amounts of chitin. Six-week-old R_1 tomato plants containing pMON8896 were sprayed with a *P. infestans* spore suspension. Five days after inoculation, water-soaked prenecrotic and necrotic spots were visible on all inoculated leaves. Histochemical staining of these leaves revealed GUS activity in mesophyll cells surrounding the necrotic lesions (Figure 3D), but no GUS activity was observed around prenecrotic spots. Similarly, no GUS activity was detected around large coalescing lesions 10 days after inoculation, suggesting that promoter activation by *P. infestans* is transitory and limited to early necrotic lesion development.

Effect of Salicylic Acid and Ethylene on GUS Expression

Exogenously added salicylic acid induces expression of a number of PR proteins, including the acidic class III chitinase in cucumber (Metraux et al., 1989). In addition, endogenous salicylic acid increases in cucumber in response to tobacco necrosis virus and Colletotrichum infection (Metraux et al., 1990), suggesting that salicylic acid functions as a transduction signal during pathogen invasion.

To test the effect of salicylate on the acidic chitinase-GUS transgene, leaves from R_1 Arabidopsis plants containing a single insertion of pMON8896 were infiltrated with 0.1, 0.5, or 1.0 mM K-salicylic acid, pH 6.7, or water. Leaves were detached and floated on the same solutions for 48 hr and then assayed for GUS activity. Figure 4 shows that treating leaves with increasing concentrations of salicylate resulted in small increases in extractable GUS activity. Average GUS activity after infiltrating and incubating leaves with 1 mM salicylate was only twofold higher than after treating with water, and an average of fourfold higher than in untreated leaves. A substantial increase in GUS activity over untreated leaves was observed in control leaves infiltrated with water. This activation of the promoter was most likely due to detaching the leaf and subsequent senescence because similar levels of induction were also observed in detached leaves that were not water infiltrated (data not shown).

To establish the shortest promoter fragment capable of activation by salicylate, leaves from plants of two lines containing the promoter deletion constructs (a high and a low expressing line) were also infiltrated with 1 mM salicylate. The averaged levels of induced GUS activity 48 hr after infiltration is shown in Table 1. Although the levels of GUS activity in plants containing the deletion constructs decrease dramatically from the levels in plants with the 1129-bp promoter, a general trend of up to twofold induction by salicylate over water treatment occurred in all transgenic plant lines.

The expression of a number of plant chitinases has been shown to be induced by ethylene (Broglie et al., 1986; Memelink et al., 1990). Ethylene increases are also associated with plant stress, including wounding and pathogen infection. To test whether the Arabidopsis acidic chitinase promoter responds to ethylene, R_1 Arabidopsis plants from three lines (AC2, AC11, and AC13) containing pMON8896 were sprayed with a 1 mg/mL solution of ethephon. Control plants were sprayed with water. After 48 hr, leaves were harvested and assayed for GUS activity. Table 2 shows that a small increase in GUS activity was observed in ethephon-treated plants but the levels of GUS activity did not exceed the levels observed in water-treated plants. In contrast, we previously observed a 30-fold increase in the class I basic chitinase mRNA level, a strong induction, in Arabidopsis plants treated with ethephon in this manner (Samac et al., 1990). This result suggests that ethephon does not cause significant activation of the acidic chitinase promoter and that the pathogen-induced activation of the promoter is ethylene independent.

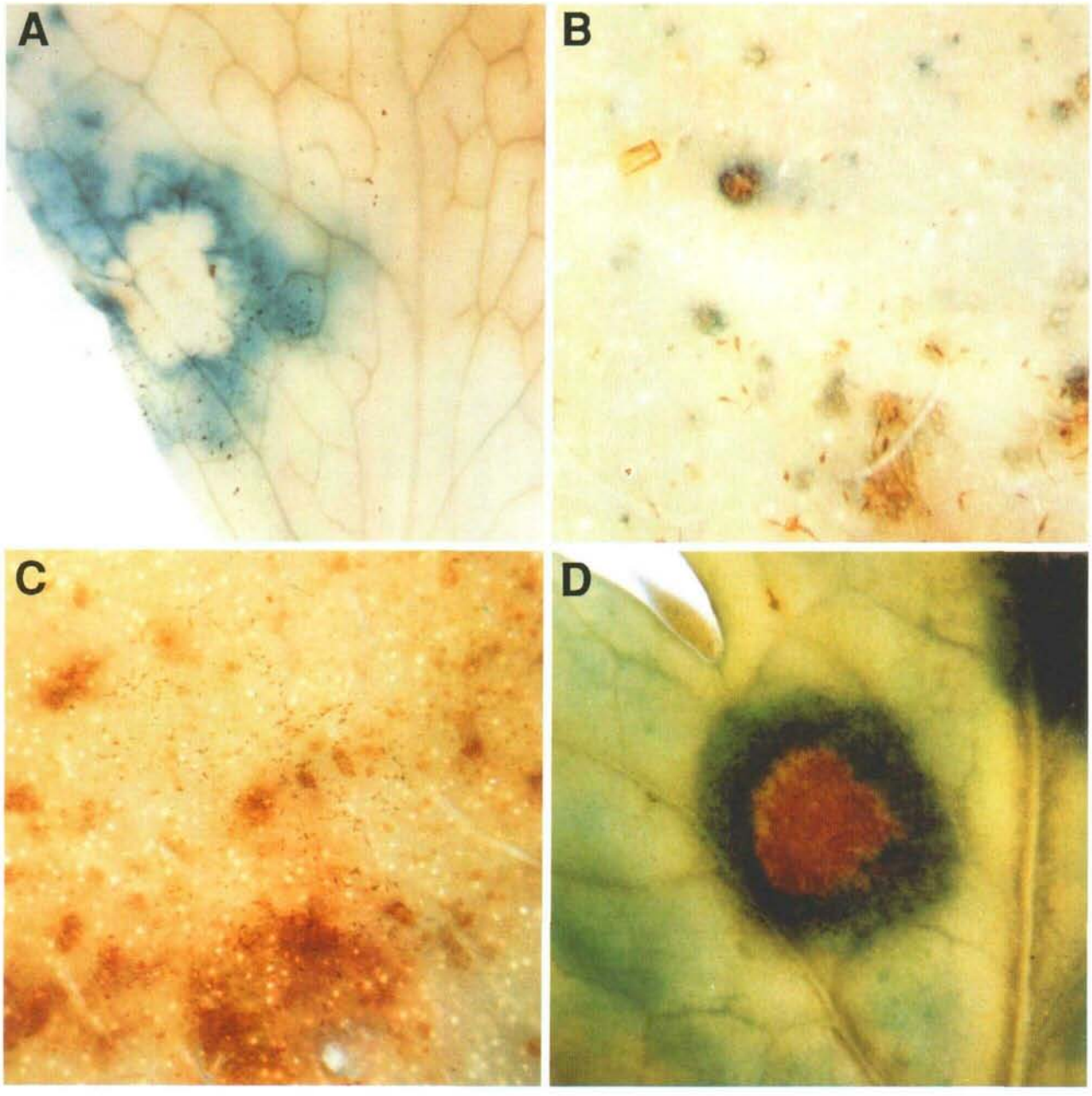

Figure 3. Histochemical Analysis of GUS Expression in Plants Containing pMON8896 Inoculated with Fungal Pathogens.

(A) GUS expression in an Arabidopsis leaf 3 days after inoculation with *R. solani*.
(B) Young tomato leaf inoculated with *A. solani* 6 days after inoculation.
(C) Old tomato leaf inoculated with *A. solani* 6 days after inoculation.
(D) Tomato leaf inoculated with *P. infestans* 5 days after inoculation.

DISCUSSION

In this paper, we report the construction of an acidic chitinase promoter-GUS transcriptional fusion and analysis of the chimeric gene expression in Arabidopsis. This approach revealed the organ and cell specificity of the acidic chitinase gene during development and in response to fungal infection. The regulation of a reporter gene by a heterologous promoter has been shown in many systems

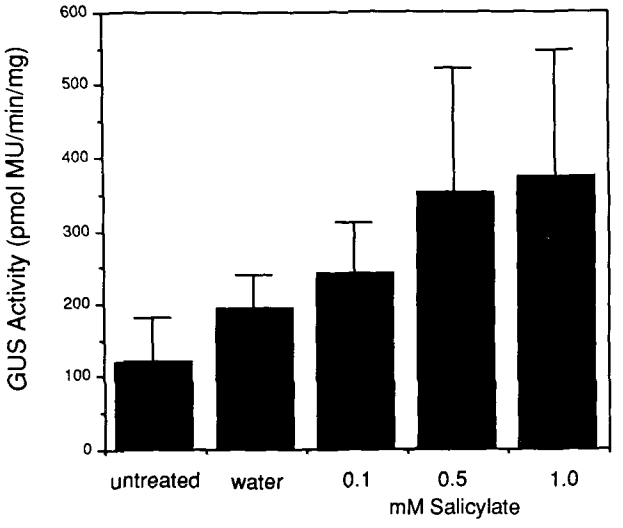

Figure 4. Effect of Salicylate Infiltration on GUS Expression.

Leaves from three Arabidopsis plants of a line containing a single insertion of pMON8896 were infiltrated with 0.1, 0.5, or 1 mM salicylic acid and then floated on the same solution for 48 hr at 24°C. Control leaves were untreated or infiltrated with water, pH 6.7. Each bar represents the mean GUS activity with standard deviation from three leaves after each treatment. MU, 4-methylumbelliferone.

to reflect accurately the intrinsic regulatory properties of the promoter. The Arabidopsis acidic chitinase promoter was active in root tissues, the leaf vascular tissue, mesophyll cells of the hydathode, guard cells of open stomates, and in anthers (Figure 1). This differential staining was not due to unequal penetration of the X-gluc staining solution because plants containing the 35S-GUS construct stained evenly.

Expression of the acidic chitinase gene in roots, hydathodes, and guard cells is consistent with the role of chitinases in plant defense. Roots are vulnerable to infection by numerous soil-borne pathogens. Opportunities for invasion by these pathogens are created by the constant wounding young roots suffer as they move through the soil and by their lack of lignified structural barriers. Thus, as a defense against invasion, healthy roots may constitutively express high levels of hydrolytic enzymes such as class I chitinases and β-1,3-glucanases (Shinshi et al., 1987; Memelink et al., 1990) and the class III chitinase we have investigated. Hydathodes are essentially open pores in leaves, which permit discharge of excess water from the plant, but lack structural barriers against pathogens. A number of pathogens, primarily bacteria, such as *Xanthomonas campestris* pv *campestris*, the causal agent of black rot in cabbage, enter leaves through hydathodes. However, the most important natural openings for pathogen entry are stomates. Several downy mildews and rust fungi enter plants almost exclusively through stomates.

Table 1. Comparison of GUS Activity Induced by Salicylic Acid in Plants Containing pMON8896 or Promoter Deletion Constructs

		Average GUS Activity (pmol MU/min/mg)[a]		
Construct	Line[b]	Water	1 mM Salicylate	Fold Induction
pMON8896	H	277 ± 38[c]	639 ± 83	2.3[d]
pMON8896	L	193 ± 26	373 ± 18	1.9
pMON8896Δ590	H	64 ± 15	95 ± 17	1.5
pMON8896Δ590	L	43 ± 18	86 ± 20	2.0
pMON8896Δ384	H	202 ± 48	326 ± 16	1.6
pMON8896Δ384	L	183 ± 7	362 ± 10	2.0
pMON8896Δ192	H	17 ± 5	28 ± 7	1.6
pMON8896Δ192	L	16 ± 4	36 ± 6	2.2

[a] MU, 4-methylumbelliferone.
[b] Two lines containing each construct were assayed, a line with high (H) and a line with low (L) constitutive levels of GUS expression.
[c] Leaves from two R_1 plants were infiltrated with water or 1 mM salicylate, incubated for 48 hr, and then assayed individually for GUS activity. Mean value from two leaves is given.
[d] Indicates fold induction of salicylate-treated plants over water-treated plants.

Stomates also provide one of the main means of entry for bacterial plant pathogens. Therefore, acidic chitinase may function as a preformed defense in locations that are especially vulnerable to pathogens.

It is not yet clear if PR protein expression in flowers, including the acidic chitinase expression in anthers, is involved in plant defense or if these proteins have a different physiological role. A variety of plant pathogenic fungi and bacteria attack floral tissues and may destroy flowers entirely or replace seed contents with fungal tissue. Class I and II chitinases and a number of other PR proteins have been found in healthy flowers (Lotan et al., 1989; Memelink et al., 1990; Neal et al., 1990) but are differentially expressed in the various flower parts (Lotan et al., 1989). Although these proteins may be present as a preformed

Table 2. GUS Activity in Transgenic Arabidopsis Containing the Acidic Chitinase Promoter-GUS Construct (pMON8896) Treated with Ethephon

	pmol MU/min/mg Protein	
Plant Line	Water	Ethephon
AC 2	68 ± 19[a]	96 ± 25
AC 11	28 ± 14	54 ± 15
AC 13	50 ± 12	63 ± 14

[a] Two plants from each line were sprayed with water or ethephon. After 48 hr, four leaves were harvested from each plant, homogenized, and assayed for GUS activity.

defense against pathogens, differential expression suggests that PR proteins may also have a role in flowering and/or reproduction or that their expression is induced coincidentally by signals involved in regulating genes involved in flowering and reproduction. Lotan et al. (1989) also suggest that genes involved in normal physiology could have evolved to have an additional function in plant defense. Future experiments will address the functional role(s) of chitinase by constructing transgenic Arabidopsis expressing antisense chitinase RNA.

To test whether the Arabidopsis acidic chitinase promoter could be induced by fungal infection, we inoculated Arabidopsis plants containing the chimeric gene with *R. solani* and tomato plants containing the chimeric gene with *A. solani*. In all cases, GUS activity appeared in mesophyll cells surrounding the infection site (Figure 3). However, simply wounding plants did not induce GUS activity (data not shown). This suggests that the induction is triggered by a combination of pathogen infection and necrosis. Similar results were obtained by Metraux et al. (1988) in cucumber. They found that the class III acidic chitinase accumulated both locally and systemically when leaves were inoculated with fungal or viral pathogens causing a necrotic response. We found GUS activity was induced in transgenic tomato plants by both *A. solani* and *P. infestans*, indicating that activation of the acidic chitinase promoter is not limited to infection by chitin-containing fungi. Promoter-GUS chimeric gene expression has been used to study the expression of the bean class I chitinase in tobacco (Roby et al., 1990). GUS activity increased in parallel to chitinase expression when plants were inoculated with fungal pathogens. As we observed with the Arabidopsis class III chitinase promoter, the class I promoter activity was greatest in and around the site of infection. These results indicate that both classes of chitinases respond similarly to fungal infection.

We found that the Arabidopsis acidic chitinase promoter was weakly activated by infiltrating leaves with salicylic acid. The GUS activity in plants containing the 1129-bp promoter construct increased only 1.9- to 2.3-fold over control plants 48 hr after infiltration with 1 mM salicylate. The low apparent activation by salicylate may be due to the relatively high background activity observed, especially if activation is transitory. The exogenous application of salicylate has been shown to induce a number of PR proteins including the cucumber class III chitinase (Metraux et al., 1989). Recent evidence indicates that salicylic acid functions as the endogenous signal in local and systemic resistance to pathogen infection. After inoculating leaves of resistant, but not susceptible cultivars of tobacco with tobacco mosaic virus, salicylic acid levels increase 20-fold (Malamy et al., 1990). Similarly, in cucumber, after infection with tobacco necrosis virus or Colletotrichum, salicylic acid levels increase dramatically in the phloem (Metraux et al., 1990). If salicylic acid is the molecule in the signal transduction pathway leading from pathogen attack to defense gene expression, then the same promoter element should respond to both pathogen attack and exogenous salicylate treatment.

To begin to elucidate the functional organization of the acidic chitinase promoter and to dissect the promoter elements involved in organ specific and pathogen induction, we made several promoter deletion constructs and tested their expression in Arabidopsis. In roots, we observed a dramatic drop in GUS expression by deleting from −1129 to −590. Interestingly, plant roots containing the 384-bp promoter (pMON8896Δ384) had 3.8 times more GUS activity than plant roots with the 590-bp promoter (pMON8896Δ590), indicating that a negative regulatory element might be located between −590 and −384. Low levels of GUS activity were found in the roots of plants containing pMON8896Δ192, and these plants retained expression in all the other tissues in which the 1129-bp promoter was active: leaf vascular tissue, hydathodes, and guard cells. In addition, GUS expression was induced in these plants by fungal infection. From these results, we conclude that the 192-bp promoter contains the elements necessary for constitutive and inducible expression. Whether expression in each tissue requires a separate promoter element and whether the same or different elements respond to salicylic acid and fungal infection remains to be determined. The step-wise reduction in the level of expression with the progressively shorter fragments suggests the presence of a number of positive regulatory elements in the 5′ upstream sequence. However, we cannot rule out the possible existence of multiple redundant elements that function additively to produce a high level of expression.

Inspection of the acidic chitinase promoter sequence did not reveal the presence of previously identified *cis*-element motifs. The promoter does not contain any sequences similar to as-1, the *cis*-acting element that has been shown to be necessary and sufficient for root-specific expression of the CaMV 35S promoter (Lam et al., 1989). The promoter from the tobacco PR protein gene, PR-1a, is induced by tobacco mosaic virus and salicylate. Van de Rhee et al. (1990) have defined a 46-bp sequence within the PR-1a promoter that responds to both tobacco mosaic virus and salicylate. A comparison of this PR-1a promoter region to the acidic chitinase promoter also failed to reveal obvious sequence similarities. Thus, direct sequence comparisons were not sufficient for identification of *cis* elements in the Arabidopsis chitinase gene. Future work will focus on identification of protein-binding domains involved in inducible expression of the acidic chitinase promoter.

METHODS

Construction of Chimeric Genes

The 5′ upstream sequence of the acidic chitinase gene was isolated from pMON8815 (Samac et al., 1990) by introducing a

BglII site just upstream of the translation initiation codon by site-directed mutagenesis (Kunkle et al., 1987). The 1129-bp SalI-BglII fragment containing the putative promoter was inserted into a promoterless expression cassette (pMON10018), consisting of the GUS coding region followed by the nopaline synthase poly(A) addition region in pUC119. The chimeric gene was excised as a NotI fragment and ligated into the unique NotI site of the plant binary expression vector pMON886 to produce pMON8896. The vector pMON886 contains the T-DNA right border sequence, spectinomycin resistance marker, and NPT II gene driven by the CaMV 35S promoter for selection of transformed plants.

Progressive 5′ end point deletions in the putative promoter sequence were generated by PCR. The upstream primers consisted of the recognition sequence for SalI and 17 bp of the promoter sequence, whereas the downstream primer contained the BglII recognition sequence and 17 bp of promoter sequence. PCR was carried out using reagents from the GeneAmp PCR Reagent Kit (Perkin-Elmer Cetus), 0.5 μg of each primer, 1 ng of pMON8815, and the standard components suggested by the manufacturer. Amplification was performed for 25 cycles of 2 min at 90°C, 1 min at 50°C, and 2 min at 72°C. The PCR products were treated with the Klenow fragment of DNA polymerase I and dNTPs, then gel purified, digested with SalI and BglII, and finally ligated into pMON10018 as above. Deleted promoters were sequenced and the promoter-GUS-NOS 3′ cassette ligated into pMON886 as above.

All binary vectors were transferred from *Escherichia coli* DH5αF′ to *Agrobacterium tumefaciens* ABI by triparental mating (Horsch and Klee, 1986). The ABI strain is *A. tumefaciens* A208 carrying the disarmed pTiC58 plasmid pMP90RK (Koncz and Schell, 1986).

Plant Transformation

Arabidopsis thaliana ecotype RLD was transformed by the root explant method essentially as described by Valvekens et al. (1988), except that vancomycin was replaced with 100 mg/L carbenicillin.

Tomato cv UC82B was transformed by the leaf disc assay as described previously (McCormick et al., 1986).

Histochemical Staining

Arabidopsis seeds harvested from primary regenerants (R_0) were surface disinfested and sown on germination medium containing 50 mg/L kanamycin (Valvekens et al., 1988). After 2 weeks, kanamycin-resistant plants (R_1) were transferred to soil (Terralite 350, Grace Horticultural Products, Cambridge, MA) in a growth chamber maintained at 24°C, 16 hr light/8 hr darkness per day, and 50% relative humidity. Tissues were vacuum infiltrated with X-gluc (5-bromo-4-chloro-3-indolyl β-glucuronide) staining solution containing 1 mg/mL X-gluc, 100 mM $NaPO_4$, pH 7, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 10 mM EDTA, and incubated for 4 to 16 hr at 37°C. Tissues were then fixed in 42% ethanol, 5% glacial acetic acid, 10% formalin.

Fluorometric GUS Assay

GUS activity in crude plant extracts was determined as described by Jefferson et al. (1987) using 4-methylumbelliferyl β-D-glucuronide as substrate and 4-methylumbelliferone to calibrate the Hoefer DNA Fluorometer TKO 100. Protein content was measured by the Bio-Rad assay using BSA as a standard.

Salicylic Acid and Ethephon Treatments

Fully expanded leaves were infiltrated from the abaxial side with 0.1, 0.5, or 1.0 mM K-salicylate, pH 6.7, using a 1-mL syringe. Control leaves were infiltrated with water, pH 6.7. Infiltrated leaves were floated in covered petri dishes on the same solution of salicylate or water at 24°C with 16 hr light/8 hr darkness for 48 hr and then assayed for GUS activity.

For ethephon treatments, 4-week-old plants were sprayed with a 1 mg/mL solution of ethephon as described by Samac et al. (1990). After 48 hr, leaves were removed and assayed for GUS activity.

Pathogen Inoculations

Four-week-old transgenic Arabidopsis plants were inoculated with *Rhizoctonia solani* by placing 1 cm^2 agar plugs of mycelium on the soil near plants. Rhizoctonia (a gift from A. J. Slusarenko, University of Zurich) was grown on potato dextrose agar at room temperature. Inoculated plants were enclosed in a plastic bag and maintained in the growth chamber as above. After 3 days, infected leaves were stained for GUS activity.

Leaves from transgenic tomato plants containing pMON8896 were excised, placed in moist chambers, and inoculated with 20 μL drops of *Alternaria solani* spores (2000 spores/mL). Inoculated leaves were incubated at 18°C in darkness for 48 hr and then transferred to a growth chamber with a 12-hr photoperiod and 24°C day, 20°C night temperatures.

Tomato plants were sprayed with a suspension of *Phytophthora infestans* sporangia at 10,000 spores per milliliter and then maintained in a dark mist chamber at 16°C for 48 hr. Plants were then transferred to a growth chamber with a 12-hr photoperiod and 21°C day, 18°C night temperatures.

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