

Analysis of Stress-Induced or Salicylic Acid-Induced Expression of the Pathogenesis-Related 1a Protein Gene in Transgenic Tobacco

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The *cis*-acting elements for regulating gene expression of the tobacco pathogenesis-related 1a protein gene were analyzed in transgenic plants. The 5'-flanking 2.4-kilobase fragment from the pathogenesis-related 1a protein gene was joined to the bacterial β -glucuronidase gene and introduced into tobacco cells by *Agrobacterium*-mediated gene transfer. Promoter activity was monitored by quantitative and histochemical assay of β -glucuronidase activity in leaves of regenerated transgenic plants. The level of β -glucuronidase activity was clearly increased by treatment with salicylic acid, by cutting stress, and by local lesion formation caused by tobacco mosaic virus infection. Cytochemical studies of the induced β -glucuronidase activity revealed tissue-specific and developmentally regulated expression of the pathogenesis-related 1a gene after stress or chemical treatment and after pathogen attack. To identify the *cis*-acting element more precisely, a series of 5'-deleted chimeric genes was constructed and transformed into tobacco plants. Transgenic plants with a 0.3-kilobase fragment of the 5'-flanking region of the pathogenesis-related 1a gene had the same qualitative response as those with the 2.4-kilobase fragment upon treatment with salicylic acid or infection with TMV. Thus, the 0.3-kilobase DNA sequence fragment was sufficient to allow the regulated expression of the pathogenesis-related 1a gene.

INTRODUCTION

Hypersensitive tobacco plants carrying the N gene can localize tobacco mosaic virus (TMV) infection to a limited area, resulting in the formation of local lesions on the leaves. With this pathological phenomenon, a set of plant-coded proteins called the pathogenesis-related (PR) proteins are induced around the necrotic lesions. In tobacco, more than 10 different PR proteins can be resolved and classified by electrophoretic analysis (Van Loon, 1985).

Acidic PR proteins of tobacco are subdivided into groups. The first, named the PR1 group (PR1a, PR1b, and PR1c), has the highest electrophoretic mobility in native polyacrylamide gels. These low molecular mass proteins (15 kD) closely resemble one another in biochemical and immunochemical properties (Matsuoka and Ohashi, 1984; Antoniw et al., 1985; Hooft van Huijsduijnen et al., 1985). Another PR protein group contains the proteins that have β -1,3-glucanase activity (PR-2, N, and O) (Kauffmann et al., 1987) and chitinase activity (PR-P and Q) (Legrand et al., 1987; Hooft van Huijsduijnen et al., 1987). Other groups consist of higher molecular weight PR proteins with unknown functions (PR-R and S) (Van Loon, 1982) or of basic PR proteins (Bol and Van Kan, 1988).

In the hypersensitive reaction, the PR1 proteins accumulate at very high levels in the intercellular spaces of

tobacco leaves (Parent and Asselin, 1984; Ohashi and Matsuoka, 1987; Hosokawa and Ohashi, 1988). They can also be induced by treatment with chemicals, such as salicylic acid (White, 1979; Ohashi and Matsuoka, 1985) and by the stress of cutting and mechanical injury (Ohashi and Matsuoka, 1985). The accumulation of these proteins results from de novo protein synthesis (Matsuoka and Ohashi, 1986) and is regulated at the transcriptional level (Hooft van Huijsduijnen et al., 1985; Pfitzner and Goodman, 1987; Matsuoka, Asou, and Ohashi, 1988). The function of PR1 proteins is not clear; however, their synthesis during the hypersensitive response makes the PR1 protein gene a good candidate for studying the induction mechanism of this class of stress-inducible protein genes.

The nucleotide sequence of the PR1a gene of tobacco has been reported by some groups. The gene has no introns and has a typical TATA promoter sequence in the 5'-flanking region (Cornelissen et al., 1987; Ohshima et al., 1987; Payne et al., 1988; Pfitzner, Pfitzner, and Goodman, 1988). We analyzed the 5' region further by constructing chimeric genes using PR1a gene promoter regions fused to the β -glucuronidase (GUS) reporter gene.

We were able to demonstrate the inducible and regulated expression of the chimeric genes that contained 0.3 kb of the 5'-upstream sequence of PR1a gene in the tobacco transformants. Histochemical analysis of the

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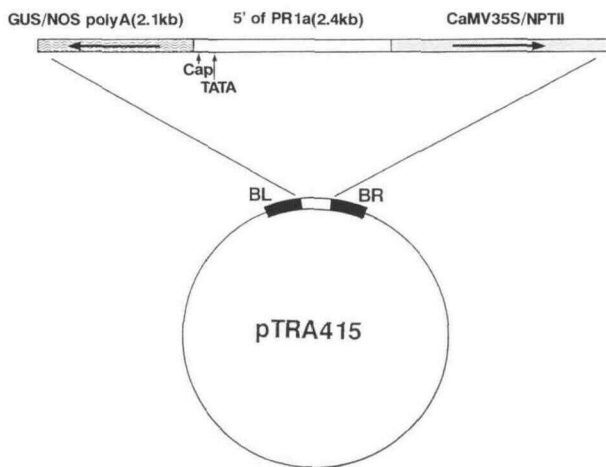


Figure 1. Structure of the Binary Vector pTRA415 Containing the PR1a(2.4 kb)/GUS Chimeric Gene.

The 2.4-kb 5'-upstream fragment of the PR1a gene was joined to the coding region of the *E. coli* β -glucuronidase gene with a NOS terminator. This chimeric gene was inserted between the left border and the selectable marker gene. BR and BL, right- and left-border sequence of T-DNA, respectively; CaMV35S, promoter fragment derived from the CaMV-35S RNA gene; NPTII, coding region for neomycin phosphotransferase II with NOS terminator. Arrows indicate the direction of transcription.

transformants showed more precisely that this inducible PR1a promoter confers tissue-specific and developmentally regulated expression.

RESULTS

Detection of the Introduced Gene in Kanamycin-Resistant Plants

An isolated 2.4-kb fragment, just upstream of the coding region of the PR1a gene, was joined to the coding region of the *Escherichia coli* GUS gene (Jefferson, Burgess, and Hirsch, 1986), with a nopaline synthase (NOS) gene terminator (Bevan, Barnes, and Chilton, 1983; Bevan, 1984) to test the promoter's activity. This chimeric gene [PR1a(2.4 kb)/GUS] was inserted into a binary vector plasmid at a restriction enzyme site located between a T-DNA left-border sequence and a kanamycin resistance selectable marker gene, as shown in Figure 1. The chimeric genes were introduced into tobacco plants by the *Agrobacterium* leaf-disc cocultivation method (Horsch et al., 1985). Resulting kanamycin-resistant (Km^r) plants were examined for the presence of an integrated intact copy of the PR1a(2.4 kb)/GUS gene by DNA gel blot analysis. The

hybridization to a 4.5-kb EcoRI fragment indicated both the proper size and approximate copy number of the gene. Those plants determined to have a single copy of the gene (E-2, E-4, E-6, E-10, and E-14) based on hybridization with DNA standards equivalent to 1, 5, and 10 copies per haploid genome (1c, 5c, and 10c in Figure 2 based on a haploid genome size of 2×10^9 bp for tobacco) showed a 3:1 segregation ratio of Km^r to kanamycin-sensitive in progeny of self-pollinated plants. A faint high molecular weight band appearing in the lane containing E-4 DNA probably is due to a partial digest or recombinant form of the introduced gene. However, this transformant also showed the same segregation ratio. Those plants containing only a single copy of the construction were chosen for the analysis of the PR1a promoter. No signal was detected in a nontransformed plant (control in Figure 2) under the same condition. Other transformants (E-1, E-5, E-9, and E-15) have two or more copies per haploid genome.

Induction of GUS Activity by Cutting or Treatment with Salicylic Acid

PR1a protein can be induced by a number of methods, among them cutting stress and salicylic acid treatment.

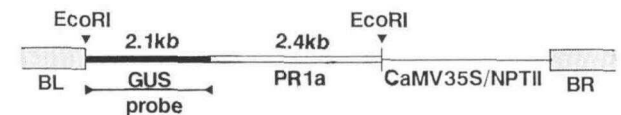
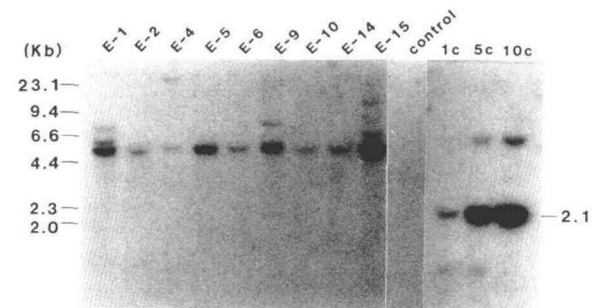


Figure 2. Detection of the Introduced Chimeric Gene in Nine Transformants.

Five μ g of EcoRI-digested high molecular weight DNA were blotted onto a nitrocellulose filter and hybridized with ^{32}P -labeled GUS coding fragment. E-1, E-2, E-4, E-5, E-6, E-9, E-10, E-14, and E-15 are the names of individual transformants; control, untransformed tobacco; 1c, 5c, and 10c, reconstruction experiment equivalent to 1, 5, and 10 copies per haploid Samsun NN tobacco genome, respectively.

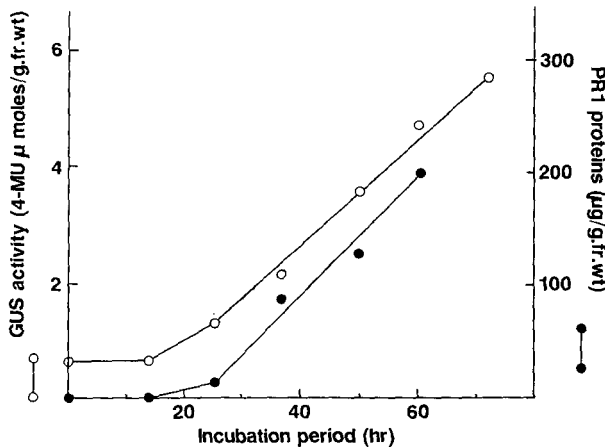


Figure 3. Time Course of Induction of GUS Activity and PR1 Protein by Treatment with 2 mM Salicylic Acid in Transformant E-3.

Leaf discs (7 mm in diameter) were floated on 2 mM salicylic acid solution under continuous light at 20°C. The level of GUS activity (○—○) and the amount of PR1 protein (●—●) were measured at the indicated times as described in Methods.

Assays of the level of induced PR1a protein by immunological tests provided an internal control for the induction levels in the transformants. As an initial study on the kinetics of PR1a induction, transformant E-3, determined to have a single copy of the chimeric gene by DNA gel blot analysis with an internal marker (data not shown), was chosen to establish the rate of accumulation of PR1a protein and to determine whether induced GUS activity followed the same kinetics. Discs (7 mm in diameter) were cut from a mature tobacco leaf and floated on 2 mM salicylic acid solution or water under continuous light of 6000 lux at 20°C. Figure 3 shows the time course of both induced GUS activity and PR1 protein accumulation after treatment with 2 mM salicylic acid. PR1 protein was not detected at time 0, the normal healthy state. By 24 hr after the treatment, considerable amounts of this protein were induced and continued to accumulate until after 60 hr of treatment. The rate of increase in GUS activity in this transgenic plant closely paralleled that of the PR1 protein. Despite a higher than expected background level at time 0, the 2.4-kb promoter sequence conferred a slight increase in GUS activity at 12 hr after treatment, followed by a dramatic increase in the rate of accumulation during the period from 24 hr to 72 hr. When assays of the other transformants containing the 2.4-kb promoter region were performed 2 days after treatment, the reactions were still in the linear portion of the response curve.

Figure 4 shows five other examples of the salicylic acid induction of GUS activity in plants with a single copy of the PR1a(2.4-kb)/GUS chimeric gene. Considerable GUS

activity [1 to 4 μ mol of 4-methylumbelliferone (4-MU)/g, fresh weight] was detected in these plants at time 0, the normal healthy state, as well as in transformant E-3 in Figure 3. Nonetheless, GUS activity was induced twofold to fivefold over that background only by cutting and subsequent incubation in media without salicylic acid. Treatment with 2 mM salicylic acid increased GUS activity to higher levels, reaching at least 10 times the background level. The salicylic acid-induced increment of GUS activity was observed in all plants that have one copy of the PR1a(2.4 kb)/GUS chimeric gene (6 to 42 μ mol of 4-MU/g, fresh weight). In transformants that have two or more copies of the PR1a(2.4 kb)/GUS chimeric gene, GUS activity was also increased by salicylic acid treatment (6 to 134 μ mol of 4-MU/g, fresh weight). However, the relationship between copy number and induction level of GUS activity was not clear. There did not seem to be any difference in the basal or induced GUS expression between transformants of cv Petit Havana SR1, which does not have the N gene, and cv Samsun NN, which does, indicating that the N genetic background does not affect the PR1a promoter. In the case of Samsun NN, GUS activity of the transformants (13 plants) was 0.2 to 8 μ mol of 4-MU/g, fresh weight, at time 0 and 1 to 71 μ mol of 4-MU/g, fresh weight, after 2 days of incubation with 2 mM salicylic acid. GUS activity in nontransformed control plants was very low and unchanged by salicylic acid treatment (0.002 to 0.01 μ mol of 4-MU/g, fresh weight).

We assayed GUS activity in 25 transformants with transcription directed by the cauliflower mosaic virus-35S pro-

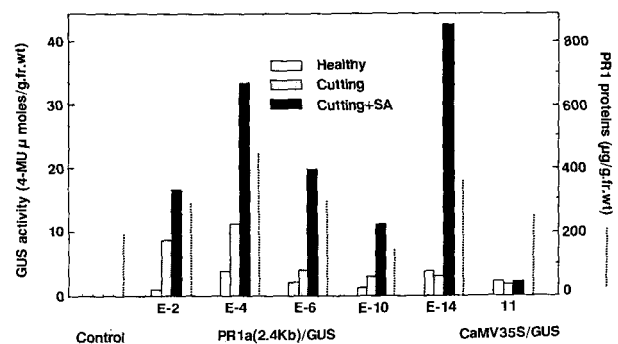


Figure 4. Comparison of β -Glucuronidase Activity Induced by Cutting and Salicylic Acid Treatment in Transformed Plants.

Leaf discs were cut from individual plants and then incubated with water or 2 mM salicylic acid. The level of GUS activity was assayed at time 0 (nontreated healthy state, white bar), after 2 days incubation with water (shaded bar) or salicylic acid (black bar). The amount of PR1 protein 2 days after induction was also measured, and is indicated by the broken line. Control, untransformed plants; E-2, E-4, E-6, E-10, and E-14, the transformants containing a single copy of PR1a(2.4 kb)/GUS; 11, transformant containing a single copy of CaMV35S/GUS.

moter [pBI121 (Clontech Laboratories, Inc., Palo Alto, CA), referred to as CaMV35S/GUS in this report] as a control for constitutive expression. Transformant CaMV35S/GUS No. 11, representative of the group, was tested, and salicylic acid did not induce an elevated GUS activity in this plant or in any of the other 24 transformants. (In some cases, however, when very young leaves were treated, GUS activity was slightly decreased after incubation with salicylic acid, perhaps because of the toxic effect of this chemical.)

In the five PR1a(2.4 kb)/GUS transformants, PR1 proteins were not detected in nontreated healthy leaves of the plants. However, 2 days after salicylic acid treatment, the amount of these proteins increased to high levels, as illustrated by the dotted line in Figure 4.

Among the transformants, the expression level of PR1 proteins and GUS activity in most examples appeared to be closely correlated. For instance, E-4 and E-14 showed a high level of PR1 protein and a high level of GUS activity in comparison with E-2 and E-10, which showed a relatively low level induction of PR1 protein and GUS activity. The observation that most of the Km^r transformants showed inducible GUS activity that paralleled the PR1 protein levels suggested that the variation in the levels of induced GUS activity may be influenced not only by position effect but also to some degree by the physiological state (such as the degree of wounding or permeability to salicylic acid) of each transformant.

5' Deletion Experiments

To define the region necessary for regulation of PR1a gene expression, a series of 5' deletions of the chimeric gene was constructed. Fragments of 0.3 kb and 0.9 kb just upstream from the coding region of the PR1a gene were excised from the 2.4-kb fragment, fused to the same GUS coding/NOS terminator [PR1a(0.3 kb)/GUS and PR1a(0.9 kb)/GUS], and introduced into Samsun NN tobacco. The resulting transgenic plants also showed increased GUS activity upon cutting and treatment with salicylic acid after 2 days of incubation. Figure 5 shows the level of GUS activity of five transformants before and after the induction. GUS activity of 1 to 2 μmol of 4-MU/g, fresh weight, was detected in the healthy leaves of these transformants before cutting. The basal level of the enzyme was not too different from that of the PR1a(2.4 kb)/GUS plants.

Considerable induction was also detected upon cutting leaf discs and the subsequent 2 days of incubation, and higher GUS activity was again found after the treatment with 2 mM salicylic acid. The salicylic acid-induced level of GUS activity of PR1a(0.9 kb)/GUS transformants was 7 to 17 μmol of 4-MU/g, fresh weight, and of PR1a(0.3 kb)/GUS transformants was 2 to 4 μmol of 4-MU/g, fresh weight. The average level of induced GUS activity in the PR1a(0.3 kb)/GUS transformants was about 3 or 4 times

lower than that of the PR1a(2.4 kb)/GUS plants. However, the induction by salicylic acid treatment and wounding was still demonstrable in the PR1a(0.3 kb)/GUS plants. Gene constructions without any PR1a promoter sequence had undetectable levels of GUS activity in tobacco transformants (the same level as with control plants, which was not increased by salicylic acid treatment). These results showed that the 0.3-kb fragment of the 5'-flanking region of the PR1a gene was sufficient to regulate the PR1a gene expression.

Localization of Induced Expression of the PR1a/GUS Gene

The localization of the expression of the introduced PR1a(2.4 kb)/GUS, PR1a(0.9 kb)/GUS, and PR1a(0.3 kb)/GUS chimeric genes in the transgenic plants (Samsun NN) was studied by in situ staining for GUS activity. Detached leaves from the transgenic Samsun NN tobacco were inoculated with TMV and incubated at 20°C in a humid chamber under constant illumination. Two days after inoculation, local lesions could be visualized on the leaves, and they continued to develop in size over time. The lesions formed on the leaves of normal control plants and CaMV35S/GUS-transformed plants were quite similar to those on the PR1a/GUS transformants. Figures 6A to 6F show the location of GUS activity as detected by 5-bromo-4-chloro-3-indolyl glucuronide (X-glucuronide) staining of tobacco leaf discs from different transgenic plants. TMV-infected leaf discs were cut out from a nontransformed control plant (Figure 6A), from a PR1a(2.4 kb)/GUS plant

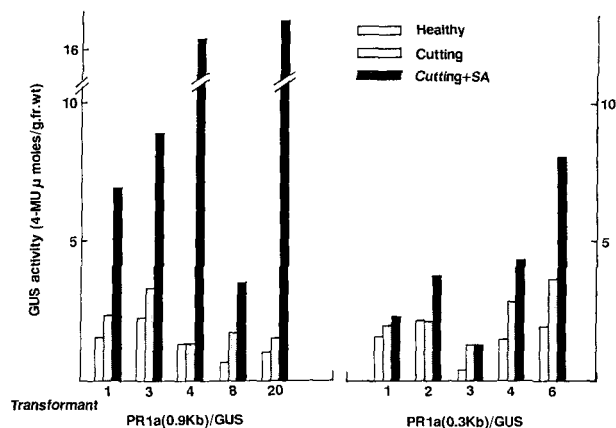


Figure 5. Comparison of β -Glucuronidase Activity Induced by Cutting and Salicylic Acid Treatment in 10 Transformants Containing 5'-Deleted Chimeric Genes.

Leaf discs were cut from transformants that have PR1a(0.9 kb)/GUS or PR1a(0.3 kb)/GUS chimeric genes. The level of GUS activity was measured as in Figure 4.

(Figures 6B, 6C, 6E, and 6F), from a PR1a(0.3 kb)/GUS plant (Figure 6D) at 3 days (Figures 6A to 6D) or 5 days (Figures 6E and 6F) after inoculation and subjected to GUS staining. No blue precipitate was detected around the local lesions in the leaf discs from the nontransformed plant (Figure 6A) or in the leaf discs from the CaMV35S/GUS transformants (not shown). A faint blue color, which cannot be seen easily in the picture, appeared in the noninfected leaf discs from a PR1a(2.4 kb)/GUS plant (Figure 6B). This weak GUS activity might reflect the leaky expression of GUS activity that corresponds to the expression level at zero time (normal healthy state without induction, white bar in Figure 4). Distinct blue rings developed around the local lesions in the leaf discs from the PR1a(2.4 kb)/GUS (Figure 6C) and PR1a(0.3 kb)/GUS plants (Figure 6D). The intensity of the staining in the leaf of the PR1a(0.3 kb)/GUS plant was always lower than that of the PR1a(2.4 kb)/GUS plant; however, the location of GUS is similar in the two plants and in the distribution of the endogenous PR1 proteins (Antoniw and White, 1986). These results confirm that the target sequence(s) responding to the stress of virus attack are contained in the 0.3-kb fragment of PR1a protein gene.

Five days after infection, a considerable level of X-glucuronide was deposited at the edge of the leaf discs of the PR1a(2.4 kb)/GUS plant, and a lower amount (which is barely detectable on the picture) was detected over the entire area of the leaf discs after overnight GUS staining (Figure 6E). This appeared to reflect the activated response to cutting stress in addition to the systemic stress of TMV infection. The induced blue color was also observed in uninfected regions distant from the local lesions in the same leaf (Figure 6F). The localization of induced GUS activity bordering the local lesions and the weak level of GUS activity over the whole leaf-disc area surrounding these local lesions after prolonged incubation, in addition to the activated sensitivity to cutting stress, were observed in all of the PR1a(2.4 kb to 0.3 kb)/GUS plants but not in any of the CaMV35S/GUS plants. This reflects the pattern of induced PR1 proteins associated with the localized acquired resistance and systemic acquired resistance responses (Ross, 1961).

In very young tobacco plants, TMV preferentially develops along small veins, resulting in local lesions with irregular form. Figure 6G shows that GUS activity was clearly detected around local lesions of TMV-infected leaves of 45-day-old PR1a(2.4 kb)/GUS-transformed Samsun NN tobacco plants, even without ethanol treatment to remove chlorophyll after GUS staining. GUS activity was not detected inside of the local lesions in this case as well as in all of the above cases, indicating that GUS protein was degraded in these dead cells.

Infection with plant pathogenic bacterium *Pseudomonas syringae* pv *tabaci* leads to the development of small local lesions 5 days after bacterial inoculation, accompanied by wide necrosis around the lesions because of the tabtoxin

produced by the bacteria. A section of the leaf, framed by the black line, from a PR1a(2.4 kb)/GUS-transformed tobacco plant (Figure 6H) was cut out and subjected to X-glucuronide staining. Dull blue rings developed around the necrotic tissue of the infected leaf (Figure 6I), whereas no induced blue color was detected in nontransformants or CaMV35S/GUS-transformed tobacco leaves after bacterial attack, indicating that *P. syringae* infection could also activate the PR1a 2.4-kb fragment promoter in this second example of pathogenic stress.

In Situ Assay of Salicylic Acid-Induced GUS Activity

Treatment with 2 mM salicylic acid causes the induction of PR1 proteins (White, 1979; Ohashi and Matsuoka, 1985). PR/GUS plants were used as a model system to analyze the localization of this induced expression by examining GUS activity. As illustrated in Figure 7, leaf discs were cut out from CaMV35S/GUS plants, PR1a(2.4 kb)/GUS plants, PR1a(0.9 kb)/GUS plants, PR1a(0.3 kb)/GUS plants, or promoterless PR1a(0 kb)/GUS plants, vacuum-infiltrated in 2 mM salicylate solution, incubated on wet filter paper at 20°C for the indicated periods, and treated with X-glucuronide to localize the GUS activity.

No blue color was observed in normal control plants (not shown), in promoterless [PR1a(0 kb)/GUS] transformants, or in the transformants without salicylic acid treatment (0 time) after GUS staining for 3 hr at 37°C. This is contrasted with the complete blue staining of whole leaf discs from PR1a(2.4 kb, 0.9 kb, and 0.3 kb)/GUS transformants induced by the 1-day treatment with salicylic acid. Longer treatment with salicylic acid induced even greater levels of blue staining. Furthermore, as depicted in Figure 7, salicylic acid treatment uniformly induces GUS activity in the leaf disc at a level greater than that of the leaf edge where cell wounding and subsequent cell damage has occurred because treatment of vacuum-infiltration is not good for the physiological condition of leaf discs.

A much fainter blue color was observed in the leaves from the CaMV35S/GUS plants, and the intensity did not change after incubation with salicylic acid. This indicates that the CaMV35S promoter, which is expressed constitutively (Odell, Nagy, and Chua, 1985), is not induced by stress or salicylic acid.

Histochemical Localization of Stress-Induced and Salicylic Acid-Induced GUS Activity

As described above, GUS activity was induced in PR/GUS plants by the stress of local lesion formation, wounding, and chemical insult. To better characterize the response and determine which tissues are involved in each of the responses, detailed histochemical studies of the tissue-specific expression of induced GUS activity were performed.

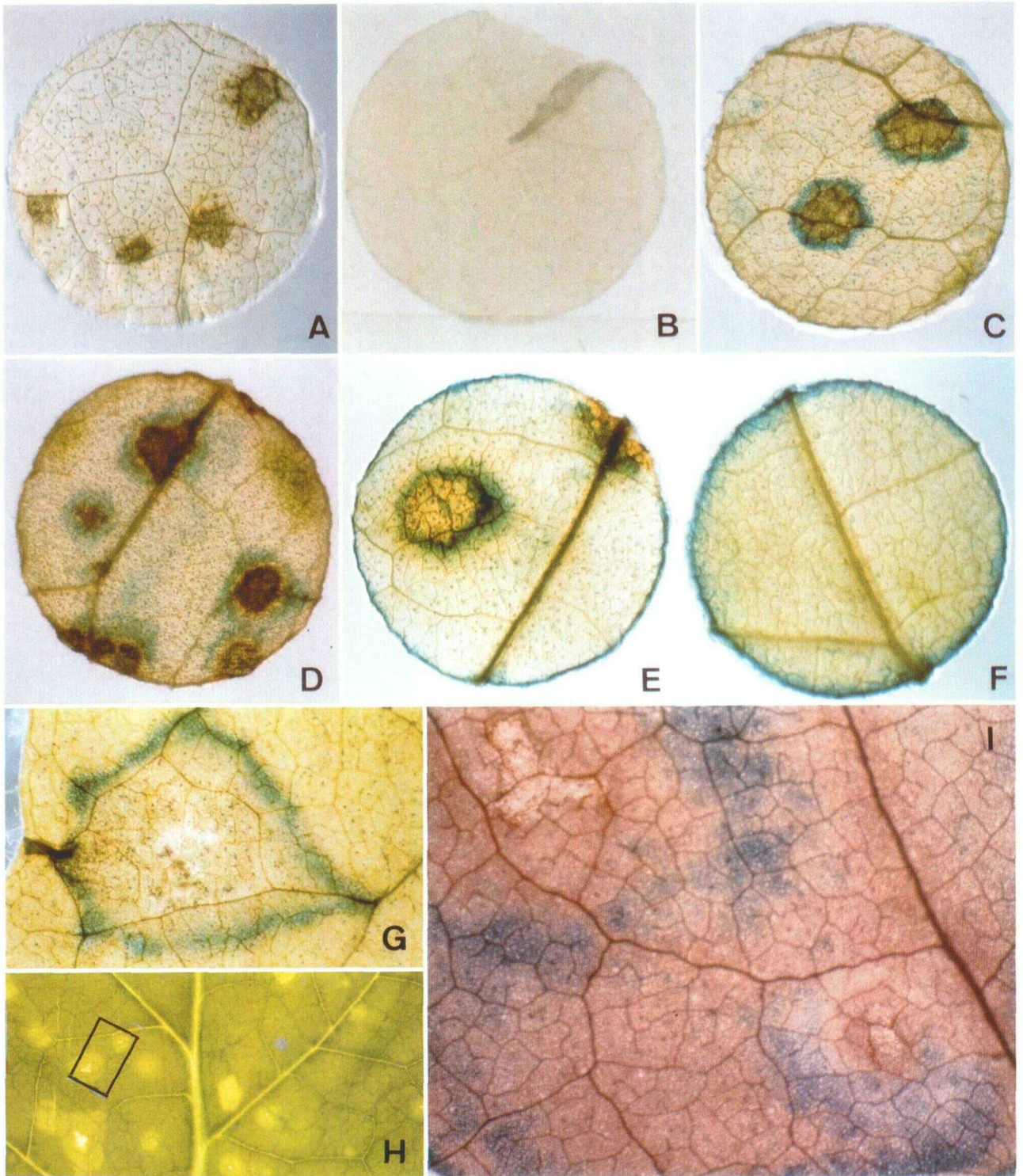


Figure 6. Localized Induction of β -Glucuronidase in PR/GUS Introduced Transformants.

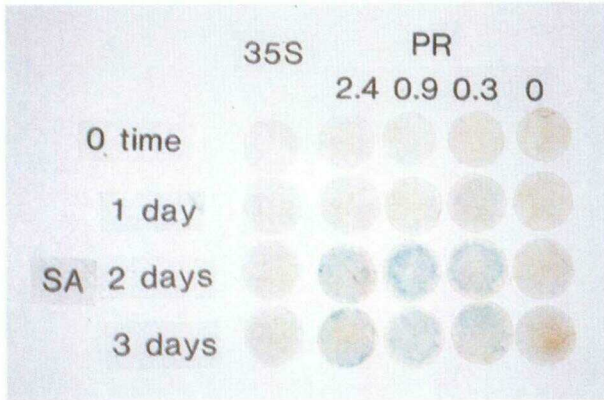


Figure 7. Localization of Salicylic Acid-Induced Expression of the GUS Gene in Transformed Plants.

Detached tobacco leaves from the CaMV35S/GUS plant (column 35S), PR1a(2.4 kb)/GUS (2.4), PR1a(0.9 kb)/GUS (0.9), PR1a(0.3 kb)/GUS (0.3), or the promoterless PR1a(0 kb)/GUS gene (0) were incubated with 2 mM salicylic acid for the indicated period. These discs were stained by the same method as described in the legend to Figure 6.

Figure 8 shows examples of the analysis. Young leaf blades from fully expanded upper leaves of the PR1a(2.4 kb)/GUS plant (Figure 8A), CaMV35S/GUS plant (Figure 8E), or a control nontransformed plant (Figure 8I) were sectioned and immediately stained *in situ* without any induction. As expected, blue color was observed in the sections from both the PR1a(2.4 kb)/GUS and CaMV35S/GUS transformants (Figures 8A and 8E) but not in the control plant (Figure 8I). GUS activity in the leaf sections from the PR1a(2.4 kb)/GUS plant was detected in all tissues of the leaf blade, e.g., mesophyll palisade cells, mesophyll spongy cells, upper and lower epidermal cells, trichome (not shown), and small vandles (Figure 8A). There

were no qualitatively clear differences in the localization of GUS activity between the transformants PR1a(2.4 kb)/GUS (Figure 8A) and CaMV35S/GUS (Figure 8E), although the intensity of blue color was stronger in the former. The expression level of GUS activity in PR1a(2.4 kb)/GUS transformants at the basal state prior to induction (Figure 8A) was the same or slightly stronger than that of CaMV35S/GUS (Figure 8E), as judged by the intensity of blue color. A similar result is also shown in Figures 3 and 4.

After induction with salicylic acid, the expression level of GUS activity in the PR1a(2.4 kb)/GUS transformant had significantly increased in all tissues of the leaf blade (Figure 8C); however, GUS levels of the CaMV35S/GUS transformant (Figure 8G) or the control (Figure 8J) nontransformed plants were not elevated (Figures 8E and 8G, 8I and 8J).

Figures 8B and 8F show the changes of expression level of GUS activity at different developmental stages of a leaf. Leaf sections were cut from fully expanded old leaves from the lower part of a PR1a(2.4 kb)/GUS plant (Figure 8B) and a CaMV35S/GUS plant (Figure 8F) without any induction. In young rigid leaf tissue (Figures 8A and 8E), GUS activity was always stronger than in an older leaf whose tissue was loosened (as seen by increasing intercellular spaces) and whose cells were thickened.

The induced level of GUS activity in these transformants with salicylic acid treatment was always higher in young leaves than in older leaves. This change of GUS inducibility is shown in Figure 9. The levels of GUS activity in the leaf discs of an old leaf or a young leaf from transformants containing PR1a(2.4 kb)/GUS, CaMV35S/GUS, or normal control plants were assayed with or without salicylic acid treatment. In the transformants containing PR1a(2.4 kb)/GUS, GUS activity was 10 times higher than the normal healthy state (time 0) in the young leaf after 2 days of incubation with 2 mM salicylic acid. In the old leaf, it increased only 3 times. In contrast, GUS activity in the transformant containing CaMV35S/GUS did not increase with salicylic acid treatment, even in the young leaf, but decreased instead. This decrease may be caused by the

Figure 6. (continued).

(A) to (G) Detached tobacco leaves were inoculated with TMV (OM strain, 0.1 μ g/mL) and incubated at 20°C. Leaf discs were vacuum-infiltrated in X-glucuronide solution, incubated overnight at 37°C, and then washed in ethanol to remove chlorophyll.

(A) Leaf disc cut from TMV-infected, nontransformed control plant 3 days after inoculation.

(B) Leaf disc cut from noninfected transformant containing PR1a(2.4 kb)/GUS.

(C) Leaf disc cut from TMV-infected transformant containing PR1a(2.4 kb)/GUS 3 days after inoculation.

(D) Leaf disc cut from TMV-infected transformant containing PR1a (0.3 kb)/GUS 3 days after inoculation.

(E) Leaf disc cut from TMV-infected transformant containing PR1a(2.4 kb)/GUS 5 days after inoculation.

(F) Leaf disc cut from uninfected region distant from the local lesions of TMV-infected transformant containing PR1a(2.4 kb)/GUS 5 days after inoculation.

(G) Young leaf from 45-day-old tobacco transformant containing PR1a(2.4 kb)/GUS 3 days after inoculation without ethanol washing.

(H) Local lesions and subsequent halos detected after bacterial infection. The leaves of a transformant containing PR1a(2.4 kb)/GUS were inoculated with *P. syringae* pv *tabaci* and incubated for 5 days in a greenhouse.

(I) The framed leaf area indicated by the black box in Figure 6H was cut out and subjected to X-glucuronide active staining.

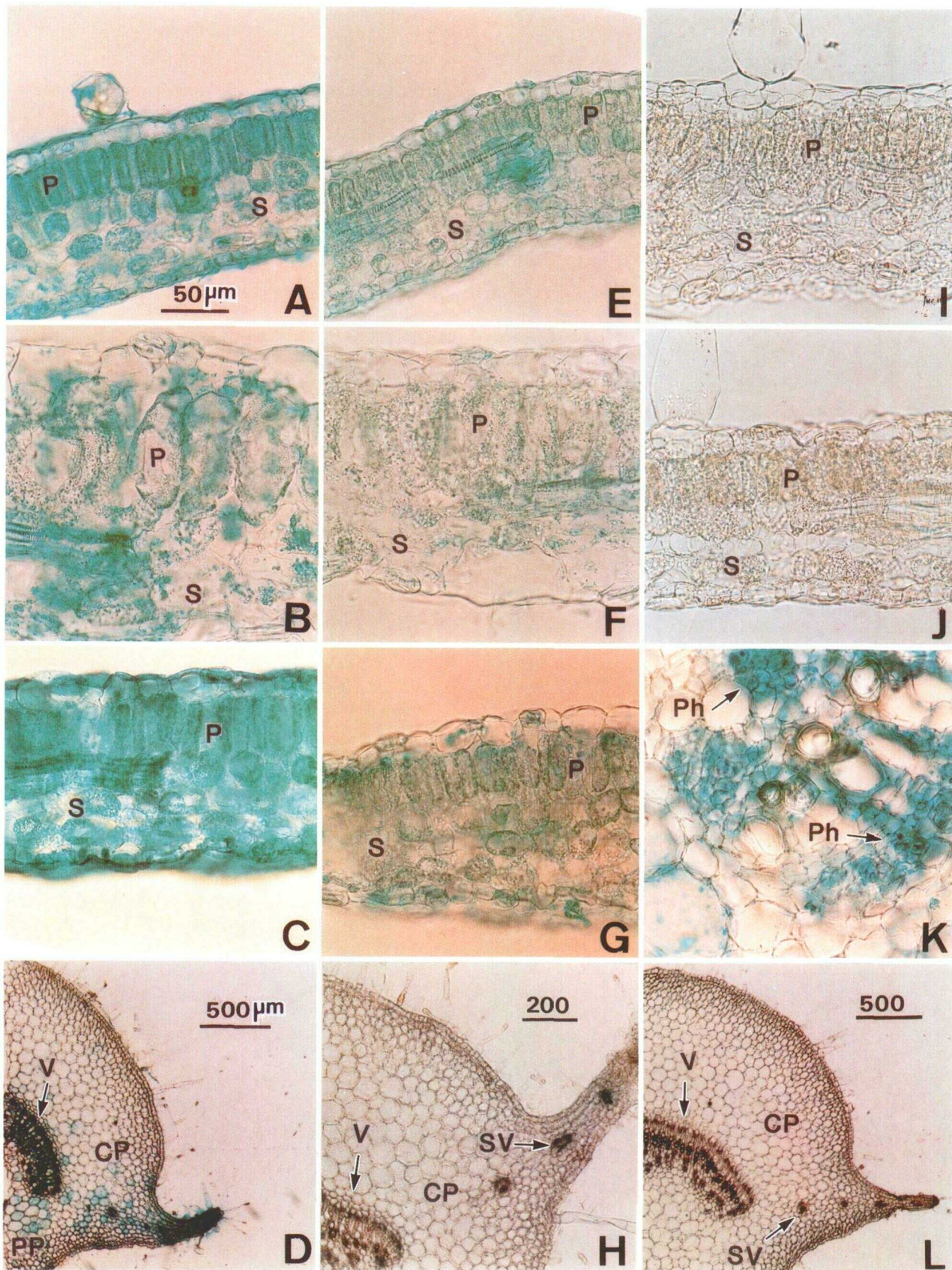


Figure 8. Histochemical Localization of GUS Activity Induced by Salicylic Acid in Leaves and Petioles of Samsun NN Tobacco Transformed with PR1a(2.4 kb)/GUS.

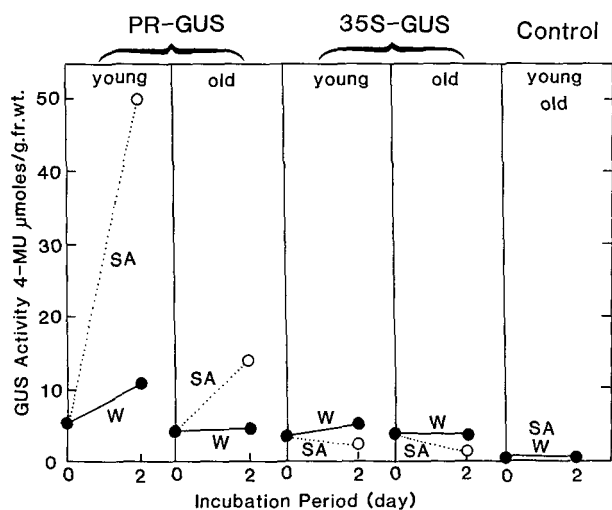


Figure 9. Changes of GUS Inducibility by Developmental Stage.

Leaf discs were cut from 2.5-month-old self-pollinated progenies of the regenerated transformants containing PR1a(2.4 kb)/GUS (PR-GUS), CaMV35S/GUS (35S-GUS), or the control nontransformed plant and incubated with water (W, solid line) or 2 mM salicylic acid (SA, dashed line) at 20°C for 2 days. A "young" leaf corresponds to an upper and fully expanded leaf; an "old" leaf corresponds to a leaf four nodes lower on the same plant.

toxicity of salicylic acid. GUS activity was not detected in the normal control plant independent of salicylic acid treatment. This result means that the PR1a promoter is more sensitive to salicylic acid in the young leaf than in the old leaf and is developmentally regulated.

GUS activity was lower in petioles than in leaves, and it could be detected only in leaf base, small bundles, phloem of young petioles from upper leaves of PR1a(2.4 kb)/GUS

transformants, and CaMV35S/GUS transformants in the basal state; no GUS activity was found in the petiole of the control plant (data not shown). Treatment with salicylic acid caused an increase of GUS gene expression in specific types of cells in the young petiole from the PR1a(2.4 kb)/GUS plant (Figure 8D). [For example, in vascular bundles, the GUS activity is located especially in phloem (Figure 8K).] In the cortical parenchyma and the pith parenchyma, a small amount of GUS activity was induced, and high activity was induced in the leaf base edge of the young petiole (Figure 8D). In old lower leaves, the induced GUS activity in petioles is always lower than that in younger leaves (data not shown), suggesting that the difference is caused by the developmental stage as well. In the young petioles of CaMV35S/GUS or control plants, no induced GUS activity was detected with the treatment of the chemical (Figures 8H and 8L).

DISCUSSION

We have transformed tobacco plants with chimeric genes consisting of PR1a 5' sequences fused to a GUS reporter gene. All of the transformants with 0.3 kb or more of the PR1a 5' sequence showed an increase in GUS activity after cutting or treatment with salicylic acid. Additionally, TMV infection and subsequent local lesion formation caused the localized and systemic induction of GUS activity. The specificity of induction and localization of GUS activity mimicked that of the endogenous PR1 proteins, thus clearly demonstrating that the 0.3-kb 5'-flanking region of the PR1a gene has sufficient activity to regulate PR1a gene expression properly.

The existence of *cis*-acting element(s) in this 0.3-kb region just upstream from the coding region can also be

Figure 8. (continued).

Fresh sections 100 μ m thick were cut with a microslicer (DTK-1000, D.S.K. Dosaka EM Co. Ltd., Kyoto, Japan), stained with X-glucuronide for 1 hr, and placed in ethanol to stop the reaction and to remove chlorophyll.

(A) Leaf section from the fully expanded upper young leaf of a 10-week-old transformant containing PR1a(2.4 kb)/GUS without any induction.

(B) Leaf section from the lower old leaf of a transformant containing PR1a(2.4 kb)/GUS without any induction.

(C) Salicylic acid-treated leaf section from a transformant containing PR1a(2.4 kb)/GUS. The leaf disc, 7 mm in diameter, was placed for 2 days at 20°C on filter paper wetted with 2 mM salicylic acid solution.

(D) Salicylic acid-treated petiole from a transformant containing PR1a(2.4 kb)/GUS. The petiole was cut in half longitudinally and put cut-surface down for 2 days at 20°C on filter paper wetted with 2 mM salicylic acid solution. Sections 100 μ m thick were then cut.

(E) Leaf section from the fully expanded upper young leaf of a 10-week-old transformant containing CaMV35S/GUS without any induction.

(F) Leaf section from the lower old leaf of a transformant containing CaMV35S/GUS without any induction.

(G) Salicylic acid-treated leaf section from a transformant containing CaMV35S/GUS.

(H) Salicylic acid-treated petiole from a transformant containing CaMV35S/GUS.

(I) Leaf section from the fully expanded upper young leaf of a 10-week-old nontransformed control plant without any induction.

(J) Salicylic acid-treated leaf section from a nontransformed control plant.

(K) Enlarged picture of (D); salicylic acid-treated petiole from a transformant containing PR1a(2.4 kb)/GUS.

(L) Salicylic acid-treated petiole from a nontransformed control plant.

Magnification of all pictures is the same except (D), (H), and (L). P, palisade mesophyll cell; S, spongy mesophyll cell; V, vascular bundle; CP, cortical parenchyma; PP, pith parenchyma; SV, small vascular bundle; Ph, phloem.

inferred from the comparison of the sequence of the PR1 protein gene family members. Approximately 0.2 kb of the 5'-flanking region just upstream of the TATA box of active PR1 protein genes (PR1a, 1b, and 1c) are highly conserved, whereas sequence insertions further upstream of the conserved region generate divergent nucleotide sequences (M. Ohshima, M. Matsuoka, and Y. Ohashi, manuscript in preparation). The fact that these PR1 protein genes are coordinatively expressed suggests that the *cis*-acting regulatory element(s) exist in this conserved 0.2-kb region.

Within this conserved region, there are two pairs of direct repeats and an inverted repeat in the TATA box region. Furthermore, overlapping this region, there is a sequence that has 70% homology to the heat shock element of the *Drosophila hsp70* gene (Pelham 1982). This degree of homology for synthetic promoters is insufficient for heat shock induction in monkey cells (Pelham and Bienz, 1982), and, in fact, PR1 proteins are not heat shock-inducible (Matsuoka and Ohashi, 1986). However, the location and resemblance to another stress-induced regulatory element suggest that this homologous structure may be important to PR1a gene expression.

The induced level of GUS activity of PR1a(0.3 kb)/GUS plants was relatively lower than that of PR1a(0.9 kb)/GUS and PR1a(2.4 kb)/GUS plants. This observation suggests that enhancing element(s) may exist between -0.3 kb and -2.4 kb.

The induction of GUS activity by salicylic acid treatment was also observed in transient expression assays. GUS activity in mesophyll protoplasts electroporated with pPR1a(0.3 kb)/GUS, as well as pPR1a(2.4 kb)/GUS, was clearly increased after the salicylic acid treatment (M. Ohshima, M. Matsuoka, and Y. Ohashi, manuscript in preparation). Furthermore, the enhancer activity of the region between 0.3 kb and 2.4 kb was also observed, as GUS expression with the pPR1a(0.3 kb)/GUS was consistently lower than with the pPR1a(0.9 kb)/GUS or pPR1a(2.4 kb)/GUS constructions.

The expression level of the PR1a(2.4 kb)/GUS gene in the transgenic plants was very high. The uninduced or background level of GUS in transformants of PR1a(2.4 kb)/GUS was similar to that of plants containing the CaMV35S/GUS (Figure 4), a promoter construct frequently used as a strong constitutive plant promoter. The reason why this level of GUS is detected at a noninduced state in PR1a/GUS-transformed plants is not obvious. We speculate that there are three possibilities: (1) position effect, (2) the constructs might have lost some other upstream or downstream regulatory region(s) that completely shut off the expression of this promoter at the basal level without any inducer, or (3) the enhancer element in the CaMV promoter sequence is present as part of the selectable marker gene on the binary vector plasmid. Further experiments will be needed to determine which possibility is the most probable. The tissue specificity observed by cyto-

chemical analysis of GUS localization in intact PR1a/GUS and CaMV35S/GUS plants was very similar (Figure 8). This might reflect the effect of the CaMV enhancer on PR1a/GUS gene expression. However, analyses after the various inducing treatments showed that the specific regulation of GUS by the PR1a sequence is not seen in the CaMV/GUS-transformed tissue.

In TMV-infected leaves, high levels of GUS activity were clearly detected only in a very narrow border around the local lesions and at low levels in a wider area some distance from them, reflecting localized and systemic induction of PR1a gene expression upon local lesion formation (Figures 6C to 6F). These observations may support the hypothesis that induced systemic gene expression is mediated by a signal produced around local lesions that is translocated to the neighboring or distant region of the plant. The mechanism of localized and systemic acquired resistances (Ross, 1961) to pathogens or stresses remains unclear, but the PR1a/GUS transformants may serve as a system to study that induction mechanism.

Histochemical analysis after salicylic acid induction of PR1a promoter activity showed that GUS activity was induced in all tissues of the leaf blade and vascular bundle, especially phloem, cortical parenchyma, pith parenchyma, and leaf base of the petiole. This induction of gene expression was also affected by the developmental stage of the leaf.

The histochemical data confirm and extend the importance of the 5' 0.3-kb flanking sequence of the PR1a gene, and show that it includes the *cis*-acting element(s) that confer tissue-specific and developmentally regulated expression by many kinds of inducing conditions, such as salicylic acid treatment, the stress of cutting, or hypersensitive reaction after infection by a plant pathogen.

The quality and quantity of the induced expression of GUS activity by stress or salicylic acid are stably inherited in progeny after as many as three generations. The strong and regulated induction using the PR1a 5'-flanking region might be valuable for practical use. For example, antisense RNA transcripts or coat protein genes of plant viruses that are joined to a PR1a promoter sequence might result in antipathogenic activity in the same regions where the PR1 proteins are induced by pathogen attack. This goal, and that of dissecting the mechanism of PR1a gene induction, will be approached by more precise studies of tissue specificity and induction kinetics using the PR1a/GUS transformants.

METHODS

Isolation of the 5'-Upstream Sequence of the PR1a Gene

A chromosomal gene of PR1a was cloned in the plasmid pBluescript SK(+) as described in a previous report (Ohshima et

al., 1987). This plasmid was converted to a single-stranded form by multiple infection with pKO7 following the instructions of Stratagene Inc. Eight μg of single-stranded DNA were annealed with a synthetic oligonucleotide (17-mer) corresponding to the upstream sequence of the antisense strand immediately 5' of the first ATG codon (5'-GACTATAGGA-3') in an equal molar ratio, at 16°C, for 10 min in a buffer containing 0.5 mM deoxynucleotide triphosphates. Sixteen units of Klenow enzyme were added, and the reaction was incubated at 16°C for 30 min. The resulting double-stranded DNA contained one nick just upstream of the first ATG. This nick was cleaved with 100 units of S1-nuclease for 60 min at 20°C. After phenol extraction, the DNA was made blunt-ended by Klenow treatment and recircularized using a synthetic BamHI linker (5'-CGGATCCG-3'). The resulting plasmid, named pPRpro, had a 2.4-kb fragment of the 5'-upstream region of the PR1a protein gene. This region was excised from the plasmid by BamHI and HindIII cut and ligated at the BamHI site to a β -glucuronidase/NOS terminator fragment prepared from pBI221 (Clontech Laboratories, Inc.). The resulting plasmid containing the PR1a(2.4 kb)/GUS chimeric gene was named pPR1a(2.4 kb)/GUS.

Transformation of Tobacco

The PR1a(2.4 kb)/GUS gene fusion was isolated from pPR1a(2.4 kb)/GUS and ligated to the binary vector plasmid pTRA415 (N. Murai and M. Ohshima, unpublished data; Figure 1). The pTRA415 has a 1.5-kb fragment of left border and a 1.2-kb fragment from the right-border sequence of the T-DNA of the octopine type Ti plasmid pTi15955. The selectable marker gene consisted of the 0.8-kb fragment of the CaMV-35S RNA promoter (derived from pBI121) fused to an NPT II gene followed by the NOS terminator sequence. The resulting construction was transferred to *Agrobacterium tumefaciens* strain LBA4404 (Ooms et al., 1981) by triparental mating. Transconjugants were checked by drug resistance and used to transform tobacco cells [*Nicotiana tabacum* cv Petit Havana SR1 (nn) or cv Samsun (NN)] by the leaf-disc cocultivation method (Horsch et al., 1985). Leaf discs were immersed in a bacterial solution and cultured on a Murashige-Skoog medium containing indole acetic acid (0.1 ppm) and benzyl amino purine (1 ppm) without antibiotics for 1 week under continuous light at 27°C. After 1 week, leaf discs were transferred to the same medium containing 500 $\mu\text{g}/\text{mL}$ carbenicillin and 300 $\mu\text{g}/\text{mL}$ kanamycin for selection of transformants. The concentration of kanamycin was reduced to 100 $\mu\text{g}/\text{mL}$ after 2 weeks. As a positive control, a chimeric gene consisting of GUS fused to the CaMV35S/GUS promoter (pBI121, referred to as CaMV35S/GUS in this report) was introduced into tobacco plants by the method described above. The shoots that formed in the kanamycin-containing medium were transferred to hormone-free MS medium for root formation. Regenerated Km^r plants were potted and cultivated in an isolated and temperature-controlled greenhouse.

DNA Gel Blotting

High molecular weight DNA was isolated from all Km^r transformants by the method described in our previous paper (Ohshima et al., 1987). DNA gel blotting was done by the standard method

(Maniatis, Fritsch, and Sambrook, 1982). Blots were washed with 0.3 M NaCl containing 0.03 M sodium citrate at 42°C for 3 hr.

Quantitative Determination of PR1 Proteins

The content of PR1 proteins was assayed by rocket immunoelectrophoresis using the antibody directed against highly purified PR1a protein. As a standard, purified PR1a protein was used as described in a previous paper (Ohashi and Matsuoka, 1985).

GUS Assay

GUS activity was assayed by the method described by Jefferson, Kavanagh, and Bevan (1987). Leaf tissue was homogenized with an aliquot of lysis buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 10 mM β -mercaptoethanol, 0.1% Triton X-100, and 0.1% sarcosyl in a small tube using a glass rod and sea sand. The homogenate was centrifuged at 10,000 g for 10 min, and the supernatant was used for GUS assay. The fluorometric reaction was carried out in 1 mM 4-methylumbelliferyl glucuronide (Clontech Laboratories, Inc.) in lysis buffer by adding an aliquot of supernatant at 37°C (400 μL total volume), and the reaction was terminated at zero time and at 30 min with the addition of 1 mL of 0.2 M Na_2CO_3 to each 200 μL of reaction mixture. Fluorescence was then measured with excitation at 365 nm and emission at 455 nm on a spectrofluorometer (model RF-540, Shimadzu Co. Ltd., Kyoto, Japan) with slit width at 10 nm. The fluorometer was calibrated with freshly prepared 4-MU standards of 100 nM and 1 μM in the same buffer. GUS activity was expressed as 4-MU μmoles produced in 30 min at 37°C per gram fresh leaf. (Recovered protein from the leaf discs was almost constant among different samples: about 30 mg of protein/g, fresh weight, in this extraction condition.)

In Situ GUS Assay

Discs (7 mm in diameter) were cut out from TMV-infected and local lesion-formed tobacco leaves, vacuum-infiltrated in 50 mM phosphate buffer (pH 7.0) containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), and incubated at 37°C in the presence of antibiotics, essentially as described by Jefferson et al. (1987). The blue precipitate that developed indicated the presence of enzyme activity. Ethanol washes were used to stop the reaction and remove chlorophyll. The discs were then transferred to water for photographing.

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