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Regulation of Pathogenesis-Related Protein-1a Gene Expression in Tobacco

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Pathogenesis-related protein-1a (PR-1a) is a protein of unknown function that is strongly induced during the onset of systemic acquired resistance (SAR) in tobacco. The expression of PR-1a is under complex regulation that is controlled at least partially by the rate of transcription. In this study, we demonstrated that 661 bp of 5′ flanking DNA was sufficient to impart tobacco mosaic virus and salicylic acid inducibility to a reporter gene. The PR-1a promoter did not respond significantly to treatments with either auxin or cytokinin. Experiments with the protein synthesis inhibitor cycloheximide indicated that protein synthesis is required for salicylate-dependent mRNA accumulation. At flowering, the PR-1a gene was expressed primarily in the mesophyll and epidermal tissues of the leaf blade and the sepals of the flower. Several artifacts, most importantly ectopic expression in pollen, were associated with the use of the β-glucuronidase reporter gene.

INTRODUCTION

Higher plants can be induced to become resistant against a variety of pathogens either by infection with a pathogen that forms necrotic lesions or by treatment with resistance-inducing compounds such as salicylic acid (SA) or 2,6-dichloroisonicotinic acid (Ross, 1961; Kuc, 1982; Métraux et al., 1991). Tobacco mosaic virus (TMV) infection of tobacco results in the formation of small necrotic lesions, thereby preventing further spread of the virus. After lesion formation, the plant becomes resistant to subsequent infection by TMV, or a variety of bacterial and fungal pathogens (Ross, 1961; Kuc, 1982). This response of tobacco to TMV was first studied in detail by Ross (Ross, 1961, 1966) and was termed systemic acquired resistance (SAR). However, the induction of general disease resistance is characteristic of similar responses in other plants that have been described for many years and collectively referred to as plant immunity (Chester, 1933).

Some of the biochemical changes occurring during the onset of SAR in tobacco have been characterized at the level of protein accumulation (Gianinazzi et al., 1970; Van Loon and Van Kammen, 1970) and gene expression (Ward et al., 1991). Nine classes of SAR mRNAs have been shown to be coordinately induced from low levels in healthy tissue to levels as high as 1% of the total mRNA in tissues expressing resistance (Ward et al., 1991). Several of the mRNAs encode proteins with known in vitro antifungal activities such as glucanases (i.e., pathogenesis-related protein-2 [PR-2]), chitinases (i.e., PR-3 and class III), and permatins (i.e., PR-5) (Mauch et al., 1988; Roberts and Selitrennikoff, 1988, 1990; Vigers et al., 1991). A particularly interesting SAR gene family encodes PR-1a, PR-1b, and PR-1c. These highly homologous 14-kD proteins are acid soluble, resistant to protease degradation, and secreted extracellularly. Recently, transgenic tobacco plants expressing high levels of PR-1a have been shown to be significantly resistant to downy mildew (D. Alexander, R. Goodman, M. Gut-Rella, C. Glascock, K. Weymann, L. Friedrich, D. Maddox, P. Ahl-Goy, T. Luntz, E. Ward, and J. Ryals, unpublished data). Based on these observations, it has been suggested that SAR proteins are at least partially responsible for maintaining the disease-resistant state (Lawton et al., 1993; Uknes et al., 1993).

Biochemical analysis of phloem exudates isolated from plants induced to resistance has identified SA as a possible endogenous signal molecule for SAR in cucumber and tobacco (Malamy et al., 1990; Métraux et al., 1990; Yalpani et al., 1991). Exogenously applied SA induces the same set of SAR genes as biological induction, providing further evidence for SA as a signal (Ward et al., 1991). However, little is known about the mechanism of SAR gene induction. Presumably, SA is bound by a receptor and the binding triggers a signal transduction cascade that has an ultimate effect on transcription factors that regulate SAR gene expression. Recently, a SA binding activity was found in tobacco (Chen and Klessig, 1991). In studies to identify DNA sequences involved in regulating SAR gene expression, two conflicting reports on the location of the SA- and TMV-responsive elements within the PR-1a promoter

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have been published (Ohshima et al.,1990; Van de Rhee et al.,1990). In addition, Ohshima et al. (1990) concluded that the PR-1 promoter is wound inducible, whereas Van de Rhee et al. (1990) do not report a wound effect. Thus, the extent of 5′ flanking DNA sufficient for pathogen and salicylate induction or regulation by wounding is still unclear.

To address the questions raised by these studies, we have performed deletion analysis on the PR-1a promoter linked to a reporter gene encoding β-glucuronidase (GUS)(Jefferson et al.,1987). Transformed plants were assayed for the response of both the transgene and the endogenous PR-1a gene to various biological and chemical treatments. We found that 661 bases of the 5′ flanking region of the PR-1a promoter were sufficient for both SA and TMV induction, supporting the findings by Van de Rhee et al. (1990). In addition, several artifacts associated with the use of GUS as a reporter gene were identified. The most important of these is ectopic expression in pollen. Finally, in studies on PR-1a gene expression in the presence of the protein synthesis inhibitor cycloheximide (CHX), we show that low concentrations induce the gene whereas high concentrations inhibit expression of PR-1a in response to SA treatment. These results indicate that ongoing protein synthesis is required for SAR gene induction and suggest that the plant may be able to sense a minor suppression of protein synthesis and react by triggering the expression of SAR genes.

RESULTS

Basal Expression from the PR-1 Promoter

To determine the amount of 5′ flanking sequence from the PR-1a gene sufficient to confer TMV and SA inducibility, a set of deletions was constructed and fused to the GUS reporter gene. The constructs were transformed into tobacco with Agrobacterium (Horsch et al., 1985) and ~15 independent transformants of each deletion were selected. Each plantlet was vegetatively split when the plants had reached the five-leaf stage and five identical plants were propagated independently. One of these was allowed to set seed, and the remaining four were used for the experimental treatments: water, SA, Carborundum, and TMV. The water treatment was taken as an indication of the basal level expression from the promoter. All of the experiments were performed on whole plants to minimize artifacts associated with leaf discs.

The results of water treatment are shown in Figure 1. Plants containing deletions of the PR-1a promoter from −903 to −318 showed very similar distributions of GUS expression among the independent transformants. GUS levels varied from ~0.3 to 300 units (1 unit = 1 pmol of methylumbelliferone per mg per min), with averages of 48, 27, 10, 12, 30, and 13 units for the −903, −825, −700, −661, −600, and −318 deletions, respectively. The average level of expression dropped to 2 units in the −222 deletion with a range from 0.2 to 10 units and dropped further in the −153 deletion to 0.5 units with a range of 0.1 to 1.5 units. Further deletion to −73 raised the basal expression to an average of 3 units, with a range of values from 0.1 to 20 units. The increased activity in the −73 deletion relative to the −150 deletion suggests the presence of a negative regulatory element that affects basal activity in the −150 region of the promoter. The variation seen for all the lines in Figure 1 is consistent with the variation observed for other promoters in transgenic tobacco and probably results from position effects (Peach and Velten, 1991).

Effects of Carborundum, TMV, and SA on GUS Expression

Transgenic tobacco treated with TMV showed strong induction of GUS activity (13-, 10-, 28-, and 12-fold) compared to Carborundum treatment for deletion lines −903, −825, −700, and −661, respectively, as shown in Figure 2. The deletion lines −600, −318, −222, −150, and −73 had an induction of three-, one-, 1.5-, two-, and threefold, respectively. These results indicate that 661 bases of the PR-1a promoter is sufficient for high-level induction by TMV. Constructs with less than 600 bases of the 5′ flanking sequence did not confer substantial inducibility to the reporter gene.

Carborundum treatment alone resulted in some induction of GUS activity compared to the basal level in T_1 plants (Figure 2A). This could be interpreted as wound induction of the PR-1 promoter. However, in previous experiments, we never detected an induction of PR-1 protein by Carborundum treatment in nontransgenic tobacco (data not shown). To investigate this effect further, the expression of endogenous PR-1a mRNA was analyzed in two of the transgenic lines by primer extension. Figure 3A shows PR-1a mRNA accumulation in two typical −903 primary transformants. In these T_1 plants, endogenous PR-1a was induced either four- or eightfold by Carborundum

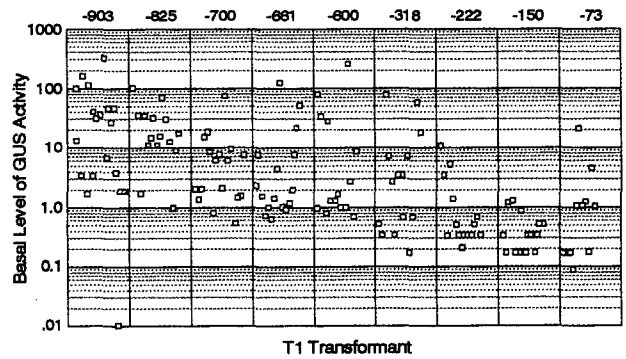

Figure 1. Basal Level of GUS Activity in Transgenic Tobacco.

Leaves from plants containing the indicated construct were treated with water and fluorometrically assayed for GUS activity. Each point represents the level of GUS activity in an individual T_1 transformant. GUS activity is in picomoles of methylumbelliferone per milligram of protein per minute (see Methods).

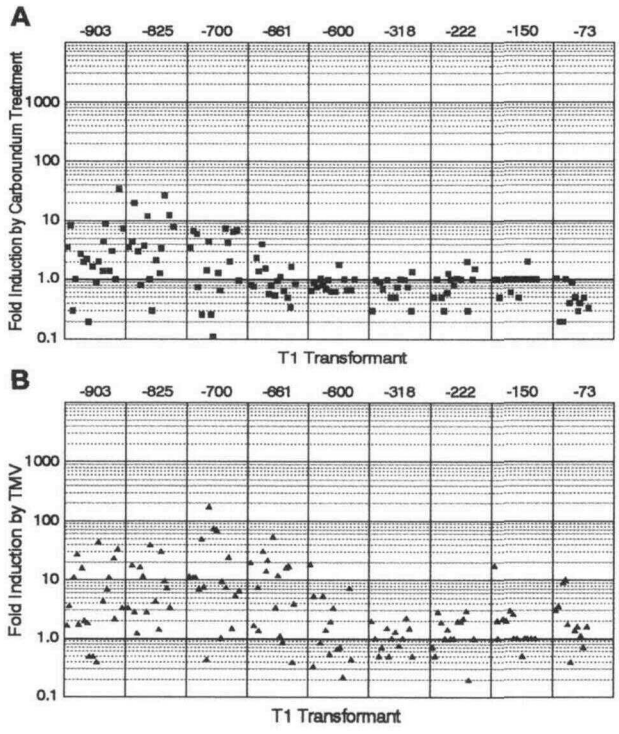

Figure 2. Induction of GUS Activity in Carborundum- or TMV-Treated Leaves of Transformed Plants.

(A) Carborundum-treated leaves (7 days after treatment) compared to the basal level.

(B) TMV-treated leaves (7 days after inoculation) compared to the Carborundum treatment.

Each point represents the level of GUS activity in a particular T_1 transformant after the experimental treatment, divided by the GUS activity of the same clone after treatment under the appropriate control conditions. GUS activity was determined fluorometrically (see Methods).

and either 20- or 40-fold by TMV. However, in homozygous T_3 lines derived from these primary transformants, Carborundum no longer induced expression of the endogenous PR-1a gene, but TMV still induced expression more than 50-fold compared to the water-treated control (Figure 3B). As indicated in Figure 3 and Table 1, this wound induction by Carborundum of both the endogenous PR-1a gene and the transgene was observed only in the primary transformants, suggesting that the result is an experimental artifact, probably due to regeneration of the plants. Interestingly, this effect maps to approximately the same promoter region that is responsive to TMV infection (Figure 2).

Because SA has been implicated as a possible inducer of SAR, we investigated the effect of SA on the PR-1a/GUS deletions. The data presented in Figure 4 indicate that SA treatment produced an average induction of greater than fivefold for deletions from −903 to −661. The −600-bp deletion line showed an average SA induction of 2.3-fold, whereas deletion lines −318 to −73 were induced 1.3-fold or less. Therefore, promoter fragments as short as 661 bases are sufficient to confer both SA and TMV inducibility to the reporter gene.

Effect of CHX on PR-1a Expression

To determine if a newly synthesized protein is required for SA induction of the PR-1a promoter, we measured the effects of the protein synthesis inhibitor CHX on gene expression. The data in Figure 5A show that a low concentration of CHX (0.01 mg/mL), which inhibited protein synthesis by less than 20% (data not shown), caused an accumulation of PR-1 mRNA. However, no accumulation of mRNA resulted from higher concentrations of CHX (0.1 and 1 mg/mL), which blocked 95 and 99% of protein synthesis (data not shown), respectively. PR-1a mRNA did not accumulate following treatment with both SA and high levels of CHX, which suggests that ongoing protein synthesis is required for induction by SA.

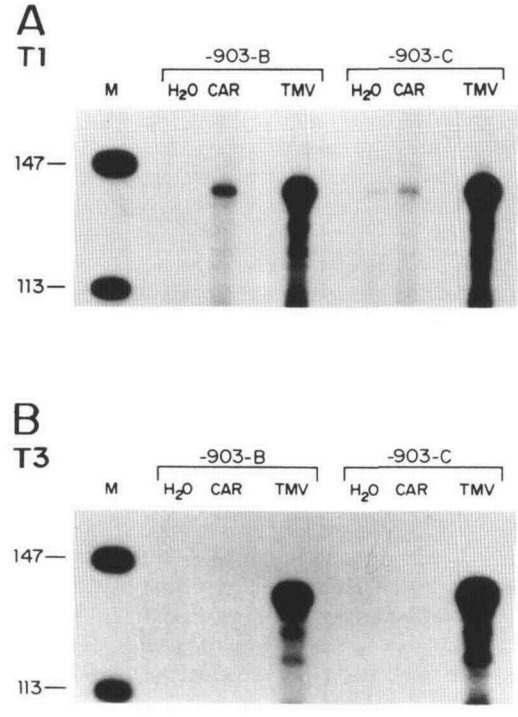

Figure 3. Primer Extension Analysis of PR-1a mRNA in T_1 and T_3 Plants.

RNA was isolated from two individual transformed lines 7 days after treatment.

(A) RNA from −903-B and −903-C in the T_1 generation.

(B) RNA from −903-B and −903-C in the T_3 generation.

The full-length primer extension product is 139 bp. Treatments were with water (H_2O), Carborundum (CAR), or tobacco mosaic virus (TMV). M, molecular size standard; size of the markers is given in base pairs.

Table 1. Average Fold Induction by TMV Infection, Chemical Treatment, and Wounding (T_3 Lines)

Construct	Carb[a]	TMV	Kinetin	BAP[b]	NAA[c]	No. of Lines
−903	1.6	56.5	1.0	0.9	1.3	9
−825	2.0	90.8	1.8	1.2	0.8	7
−318	2.4	2.1	1.0	0.9	0.8	5

[a] Carborundum.
[b] 6-Benzylaminopurine.
[c] 1-Naphthaleneacetic acid.

The effect of CHX was also determined for the T_3 lines of the −903 PR-1a/GUS fusions by primer extension (data not shown) and RNA gel blot analysis (Figure 5B). GUS mRNA abundance was induced by low concentrations of CHX (0.01 mg/mL, less than 20% protein synthesis inhibition). However, in contrast to the endogenous gene, high concentrations of CHX caused accumulation of GUS mRNA. This result was repeated in six −903 T_3 lines, all of which yielded similar results (data not shown).

Several possibilities could explain these results. First, because these lines were transgenic for the PR-1a promoter, a *trans* effect due to an increased copy number of the promoter may have caused the difference in expression. If this were indeed the case, the endogenous gene should respond in a manner similar to the transgene. Therefore, accumulation of PR-1a mRNA was evaluated in each of the −903 lines. As seen in Figure 5C, the endogenous PR-1a mRNA accumulation in the transgenic plants was essentially the same as in the nontransformed plants, eliminating a *trans* effect as an explanation.

Second, the anomalous GUS results could also be explained by the presence of CHX-responsive elements contained in the −903 promoter, which are normally silenced in the endogenous gene by the action of a *cis*-acting sequence upstream of −903. If this were the case, it should be possible to map the CHX-responsive element by analyzing CHX induction in each of the deletion lines. Six independent lines of each deletion were evaluated for CHX induction (data not shown). GUS mRNA accumulated to high levels following treatment of all the lines, suggesting that if there was a CHX-responsive element in the promoter, it had to be located between −73 and the transcriptional start site.

Another possible explanation was that CHX affected the GUS coding sequence, rather than the PR-1a promoter. To test this explanation, we analyzed two independent transgenic plants containing the −903 PR-1a promoter fused to a *Bacillus thuringiensis cry IA(b)* gene for CHX induction. In controls treated with SA, *B. thuringiensis* toxin mRNA accumulated to high levels (data not shown; Williams et al., 1992). However, CHX treatment (1 mg/mL) resulted in no mRNA accumulation (data not shown). Therefore, the simplest explanation is that CHX induction of the PR-1/GUS gene is an artifact of the GUS coding region. We have not determined whether CHX induction is a transcriptional or post-transcriptional event.

Kinetin, 6-Benzylaminopurine, and 1-Naphthaleneacetic Acid Effects on PR-1a Promoter Activity

Several groups have reported that the PR proteins could be induced by treatment with certain plant hormones (Antoniw et al., 1980; Shinshi et al., 1987; Memelink et al., 1990). To map the potential hormone-responsive regions of the PR-1a promoter, T_3 plants from deletion lines −903, −825, and −318 were treated with kinetin, 6-benzylaminopurine (BAP), 1-naphthaleneacetic acid (NAA), Carborundum, or TMV, and the induction of GUS activity relative to water treatment was determined. As shown in Table 1, no significant induction (greater than threefold) was seen for kinetin, BAP, NAA, or Carborundum treatment. However, TMV showed strong induction (greater than 50-fold) in deletion lines −903 and −825, but not the −318 line as expected (Figure 2). Figure 6 shows the results when PR-1a mRNA was measured in nontransgenic Xanthi-nc tobacco following BAP, kinetin, NAA, and SA treatment. Only SA treatment resulted in a significant increase in the level of RNA accumulation.

Tissue-Specific Expression from the −903 PR-1a-GUS Transgene

Nine T_3 lines were examined for tissue-specific expression of GUS under various conditions. Three patterns of expression were identified: consensus, constitutive, or undetectable. Five of the lines (−903-B, −903-F, −903-G, −903-H, and −903-I)

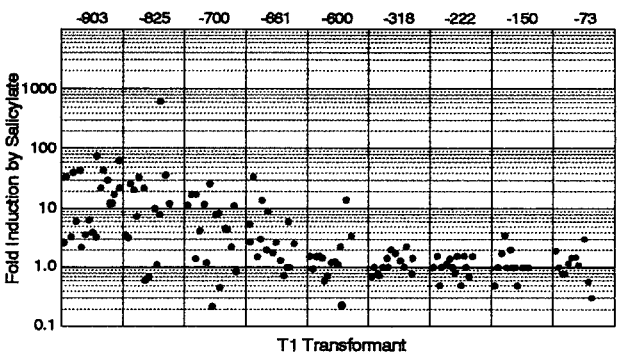

Figure 4. Induction of GUS Activity in SA-Treated Leaves Compared to the Basal Level.

Each point represents the level of GUS activity in a particular T_1 transformant after SA treatment divided by the GUS activity of the same clone after treatment with water under the appropriate control conditions. GUS activity was determined fluorometrically (see Methods).

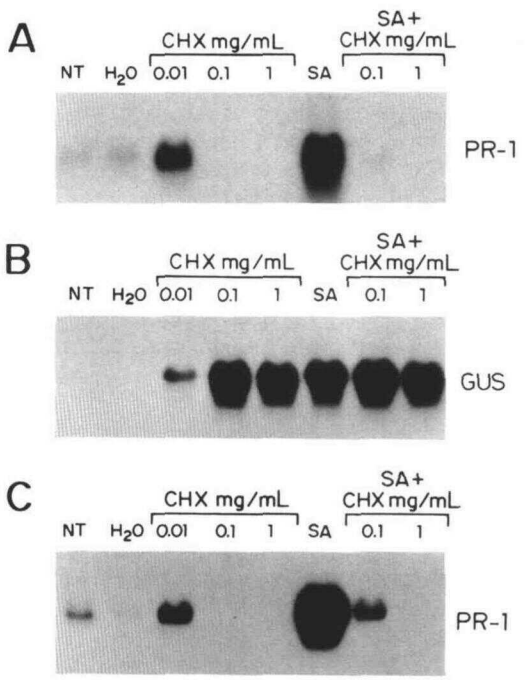

Figure 5. Effect of CHX on PR-1a and GUS mRNA Accumulation in Nontransformed and Transformed Plants.

RNA was isolated from leaves 24 hr after the treatment indicated. RNA gel blots were probed with either labeled PR-1a cDNA or labeled GUS-specific DNA. NT, nontreated tobacco; H_2O, water treated; SA, treated with 1 mM salicylic acid.

(A) PR-1a mRNA in nontransformed tobacco.

(B) GUS mRNA in transformant −903-A.

(C) Endogenous PR-1a mRNA in transformant −903-A.

showed a consensus pattern of expression summarized in Table 2. A sixth line (−903-A) mimicked this pattern but at higher levels of expression. As shown in Figure 7, the consensus pattern included high-level expression in the mesophyll layer and a moderate level of expression in the epidermal layer of the leaves in response to both SA (Figure 7A) and TMV (Figures 7B and 7C), but no induction in response to water or Carborundum (Figure 7D). There was also no GUS activity in the roots of transgenic plants following TMV infection of the leaves (Figure 7E).

The TMV induction of GUS activity appeared greatest close to the lesion (Figures 7B and 7C), similar to what has been shown for the PR-1a protein (Antoniw and White, 1986) and consistent with the work of others (Ohshima et al., 1990). However, when six independent plants from each of the deletion lines (−600 to −150) were tested for TMV and SA induction, no GUS staining was detectable (Figures 7F and 7G; data not shown). This is in contrast to the results of Ohshima et al. (1990), who found TMV-inducible GUS activity even in a 300-bp PR-1a promoter/GUS transgenic plant. It is possible that some other factor, perhaps the orientation of the PR-1a promoter relative to the cauliflower mosaic virus 35S promoter, is responsible for the observed difference in histochemical staining.

Stem sections from the consensus lines showed no or low expression in response to inducers. Petiole sections showed low levels of expression in the cortex in response to TMV and SA; however, this GUS staining was detectable only after long incubation times with the chromogenic substrate (data not shown). The −903-A line also demonstrated GUS activity in the cells surrounding the vascular bundle of the petiole (i.e., phloem) in response to SA after long incubation times (data not shown).

Two lines (−903-C and −903-E) constitutively expressed GUS activity. Regardless of the presence of inducer, GUS activity was seen in leaves, petioles, and stems (data not shown). A single line (−903-D) had low GUS activity that was inducible and detectable by fluorometric assay, but not by in situ GUS staining (data not shown).

PR-1a-GUS Expression in Flowering Plants

Several reports have indicated that PR proteins accumulate in leaf tissue during flowering (Fraser, 1981; Lotan et al., 1989). To determine if the −903/GUS plants were responding appropriately to this developmental signal, we assayed tobacco plants from the consensus set of T_3 −903 PR-1a–GUS lines for GUS expression in certain tissues of untreated flowering plants. As can be seen in Figure 7H and summarized in Table 2, the leaves of untreated flowering plants containing the −903/GUS gene showed GUS activity, whereas untreated nonflowering plant leaves showed no GUS activity (data not shown). In flower tissue, GUS activity was observed at the base of the sepals and in mature pollen grains (Figures 7I, 7J, and 7K). Other tissues tested, including roots, petals, and the female structures of the flower, exhibited no reproducible GUS activity in response to flowering (Figure 7; data not shown). Nontransformed tobacco showed no GUS activity in any tissue, including pollen (Figure 7L; data not shown). In addition,

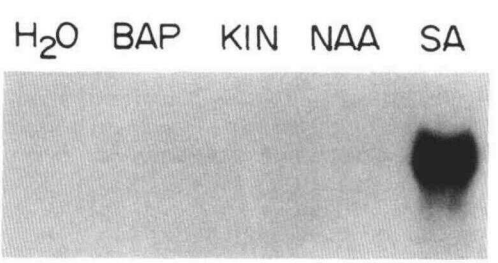

Figure 6. Effect of Phytohormones on PR-1a Expression.

Tobacco leaves were painted with the compound indicated at 1 mg/mL; kinetin (KIN), 6-benzylaminopurine (BAP), 1-naphthaleneacetic acid (NAA), and salicylic acid (SA). RNA was isolated 48 hr after treatment and probed with PR-1a cDNA on an RNA gel blot.

Table 2. Tissue Specificity of GUS Activity in −903 PR-1a–GUS Transgenic Plants

Tissue	Water	SA	TMV	Flowering
Leaf	−[a]	+ +[b,c]	+ + +[c,d]	+ + + +[c]
Petiole	−	−	+[e]	+[e]
Stem	−	−	+/−[e]	+ +[e]
Root	−	−	−	−
Pollen	nt[f]	nt	nt	+ + +
Sepal	nt	nt	nt	+ +

[a] −, No GUS activity detectable.
[b] + to + + + +, Varying degrees of GUS staining.
[c] Expressed predominantly in mesophyll cells.
[d] Expression strongest near lesions.
[e] Expressed predominantly in the cortex.
[f] nt, Not tested.

immature pollen grains of transgenic tobacco showed low or no GUS activity detectable by in situ staining (data not shown). It is difficult to determine the level of GUS activity in the anther tissues surrounding the pollen because of the possibility of contaminating GUS activity from the pollen (Figure 7L). Although PR-1 protein has been detected in sepals, perhaps associated with nectaries, it has not been detected in the pollen (Lotan et al., 1989).

To determine whether the pollen-specific expression was a reflection of the activity of the endogenous promoter, we analyzed transgenic plants for the expression of both the native PR-1a gene and the PR-1a/GUS transgene in anthers/pollen. As shown in Figure 8, expression of the endogenous gene was not detected in anthers/pollen, but the GUS gene was detectable in anthers/pollen at levels approaching that of induced leaves. RNA was also analyzed from transgenic tobacco containing the same PR-1a promoter fused to a *B. thuringiensis cry IA(b)* gene. No *B. thuringiensis* toxin mRNA was detected in the pollen from two independent transformants. However, normal SA- and TMV-inducible expression was observed in leaf tissue (data not shown; Williams et al., 1992). The simplest explanation for this result is that the GUS coding region is influencing the expression of the transgene (either transcriptionally or post-transcriptionally), causing accumulation of GUS RNA in the pollen, a tissue in which the PR-1a promoter is not normally active. Therefore, caution should be used when interpreting GUS histochemical staining when the tissue specificity of the endogenous gene is unknown.

DISCUSSION

TMV- and SA-Responsive Elements

The expression of PR-1a by viral infection and SA treatment has been studied for more than 10 years, yet an understanding of the induction mechanism is not clear. In experiments designed to identify the *cis*-acting sequences of the PR-1a promoter responsible for induction, Ohshima et al. (1990) concluded that as little as 300 bp of 5′ flanking sequence was sufficient to appropriately express a GUS reporter gene. However, Van de Rhee et al. (1990), using the same PR-1a gene from tobacco and similar reporter gene constructs, concluded that induction required at least 685 bp.

To resolve this issue, it is important to carefully study the data and the experimental procedures from both laboratories. Ohshima et al. (1990) reported induction of approximately eightfold above H_2O treatment by SA with their −900 construction and 1.5-fold with a −300 construction. They further reported that the −300 and the −900 lines were equally induced by TMV infection, but the only data supporting this claim was a histochemical analysis, which is a difficult method to interpret quantitatively. On the other hand, Van de Rhee et al. (1990) reported induction of approximately fourfold by SA and ~10-fold by TMV in a 685-bp construct and no induction in constructs containing 645 bp or less. In fact, with less than 645 bp, there was no measurable GUS activity. However, in contrast to the work by Ohshima et al. (1990), Van de Rhee et al. (1990) analyzed at least 10 independent transformants from each deletion, lending more weight to their analysis.

The observations in these studies are not mutually exclusive. The dispute lies in what each group considers to be "induction." In the study of Ohshima et al. (1990), an increase of 50% in the level of GUS activity was scored as induced, whereas in the Van de Rhee et al. (1990) study, the induction had to be at least fivefold to be scored as induced. Using the same induction criteria, the two groups would have reached similar conclusions. In support of this interpretation, using a large number of primary transformants and the sensitive fluorometric assay for GUS, we have found that 661 bp of 5′ flanking sequence is sufficient for fivefold salicylate and 13-fold TMV induction. We have extended these observations with data suggesting the presence of sequences responsible for high-level uninduced expression that lie approximately at position −300 and a possible negative regulatory element that contributes to uninduced expression near position −150.

However, it is worth noting that the endogenous PR-1a gene is typically induced ~1000- to 10,000-fold in TMV-infected or SA-treated leaves, increasing from very low levels to ~1% of the mRNA in induced tissue (Ward et al., 1991). Thus, other factors could play an important role in the regulation of PR-1a expression, which might include as yet unidentified *cis* elements in the more distant 5′ or 3′ regions of the gene as well as the location of the gene in the genome.

Artifacts Associated with GUS as a Reporter Gene

Several studies have documented GUS artifacts associated with the histochemical GUS activity assay (Plegt and Bino, 1989; Mascarenhas and Hamilton, 1992; Tor et al., 1992). In

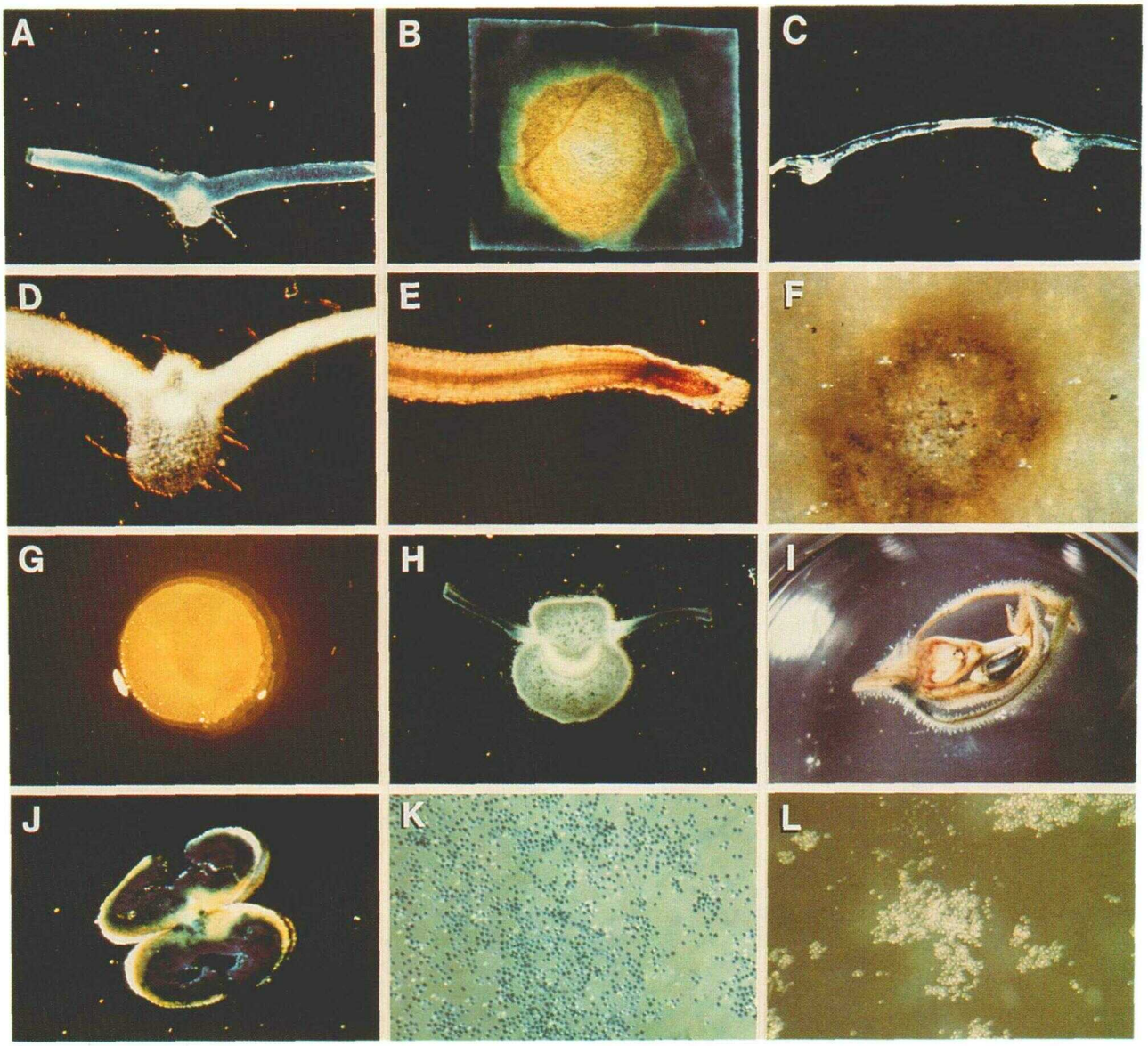

Figure 7. In Situ GUS Staining of PR-1a–GUS Plants.

(A) SA-treated −903-B leaf cross-section stained 3 days after inoculation.
(B) TMV lesion from an infected −903-F leaf stained 7 days after inoculation.
(C) TMV lesion from an infected −903-A leaf cross-section stained 7 days after inoculation. The area between the veins is a TMV lesion.
(D) Buffer-treated −903-A leaf cross-section stained 7 days after inoculation.
(E) Secondary root from −903-A in which several leaves were infected with TMV, stained 7 days after treatment.
(F) TMV lesion on −600-E, stained 7 days after infection.
(G) SA-treated −600-A leaf disc showing lack of blue staining.
(H) Petiole from flowering −903-A.
(I) Immature flower cross-section of −903-A.
(J) Anther cross-section from −903-A.
(K) −903-B pollen.
(L) Nontransformed tobacco pollen stained for GUS activity.

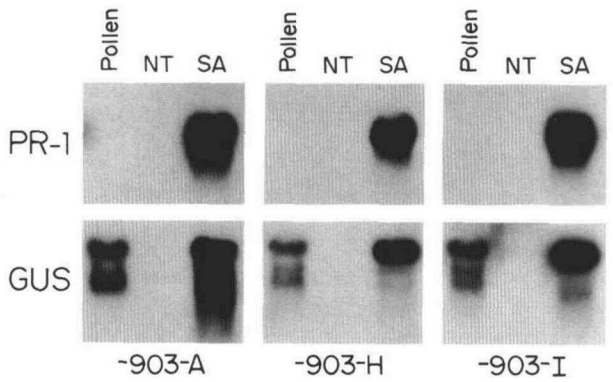

Figure 8. Pollen-Specific Expression of the −903-GUS Gene.

RNA was isolated from the −903 T_3 lines indicated. RNA gel blots were probed with PR-1a (top) or GUS (bottom). Pollen, anthers and pollen from mature and immature flowers; NT, nontreated leaf tissue; SA, leaf tissue treated with 1 mM salicylic acid for 24 hr.

this report, we have documented two artifacts associated with the use of GUS as a reporter gene in transgenic plants that are related to actual GUS gene expression and not with the GUS activity assay. We have shown that the treatment of tissue with high levels of CHX significantly increases the accumulation of GUS mRNA, but not mRNA from the endogenous PR-1a gene or from other reporter genes under the control of the PR-1a promoter. The cause of this accumulation is not clear. One explanation for the aberrant RNA accumulation could be that the GUS coding sequence contains a promoter element that responds to CHX treatment. Another possibility is that a low level of GUS transcription always occurs, but the mRNA becomes stabilized following CHX treatment. In either case, the reporter is clearly not responding in a manner consistent with expression of the endogenous gene or other genes under the control of the PR-1a promoter.

The second artifact was in the ectopic expression of GUS activity in the anther and pollen of untreated flowering plants. This expression appears to be due to the GUS coding region because other reporter genes linked to the same promoter do not show this effect. In many similar studies using GUS as a reporter gene, investigators have concluded that the promoter under study directed pollen- or anther-specific expression (Guerrero et al., 1990; DeWitt et al., 1991; McCormick, 1991; Samac and Shah, 1991; Takahashi et al., 1992). Our data indicate that these results must be reevaluated and future experiments concerning pollen expression should be carried out either directly on the endogenous gene or with several different reporter genes to minimize artifacts. Moreover, further problems not yet documented may arise from the use of GUS as a reporter gene. Thus, if GUS is not reliable as a reporter in the two cases documented here, when is it? It is clear that the GUS reporter gene should be used only when there is sufficient prior knowledge concerning the expression characteristics of the endogenous gene under study.

Effects of Protein Synthesis Inhibitors

The finding that low concentrations of CHX induced the endogenous PR-1a promoter, whereas high concentrations of CHX prevented induction of the endogenous gene by SA, suggests an intriguing model for PR-1a induction. Incomplete inhibition of protein synthesis may be one signal for PR-1a gene induction. When a plant cell is infected, the pathogen may partially inhibit host protein synthesis. Some mammalian pathogens, such as polio virus, are known to exert such an effect on their hosts (Hershey, 1991). Conceivably, the plant could have evolved a mechanism to induce defense genes when protein synthesis is partially inhibited. However, because complete inhibition of protein synthesis abolished PR-1a induction, a newly synthesized protein is apparently required for the induction of the endogenous PR-1a gene.

METHODS

5′ Deletion Constructs

An 1150-bp XhoI-PstI fragment of a pathogenesis-related protein-1a (PR-1a) genomic clone was subcloned into M13mp18, as described by Payne et al. (1988). Site-directed mutagenesis (Kunkel et al., 1987) of the resulting clone was performed at the initiation codon of the PR-1a protein, changing the sequence from TC ATG G to CC ATG G, thus creating an NcoI site. The resulting clone, designated PR-1aΔXhoNco, was used as the source of PR-1a promoter DNA for the deletion constructs.

A vector called pBSGus1.2 was created by a three-way ligation of a 391-bp SalI-SnaBI fragment from pRAJ265 (Jefferson et al., 1987), containing the 5′ end of the β-glucuronidase (GUS) coding sequence, with a 1707-bp SnaBI-EcoRI fragment from pBI221 (Clonetech, Palo Alto, CA), containing the 3′ end of the GUS coding sequence and the nopaline synthase terminator, and pBS+ (Stratagene) digested with SalI and EcoRI. An NcoI site was created at the initiation codon of the GUS gene using site-directed mutagenesis (Kunkel et al., 1987).

PR-1aΔXhoNco was digested with NcoI and other enzymes located within the PR-1a promoter sequence at varying distances upstream from the start of transcription: XhoI (−903), AluI (−825), SspI (−318), DraI (−222), BstEII (−150), and RsaI (−73). These promoter fragments were isolated; adapters were ligated at their 5′ ends and cloned into pBSGus1.2, previously digested with XhoI and NcoI.

Other 5′ deletion constructs were made using the polymerase chain reaction (Perkin-Elmer, Norwalk, CT). Primers were designed to correspond to the −700, −661, and −600 sequences of the PR-1a promoter with XhoI restriction sites on the 5′ end. The second primer for each reaction was located in the GUS coding region. The −903 construct described above was used for template DNA. Polymerase chain reaction was performed according to manufacturer's recommendations (Perkin-Elmer).

The numbering of the constructs is according to the method of Payne et al. (1988), with the transcriptional start site designated as +1 and the translational start at +29. A putative TATAA box is located at −34. The junction of the PR-1a promoter fragments with the GUS gene is at the PR-1a translational start site. All constructs were sequenced to confirm their identity.

Each of the deletion constructs was digested with KpnI-BamHI and the promoter-GUS fragment was cloned into KpnI-BamHI–digested pCIB200, a binary vector containing the neomycin phosphotransferase II gene that confers kanamycin resistance. All of the plasmids were constructed such that the PR-1a promoter was adjacent to the left border of the T-DNA, with transcription from both the PR-1a promoter and the neomycin phosphotransferase II gene in the same direction. The resulting constructs were transformed into *Agrobacterium tumefaciens* CIB542 according to the procedure of Holsters et al. (1978). CIB542 is strain EHA101 (Hood et al., 1986), in which the kanamycin marker of the plasmid has been replaced by the spectinomycin/streptomycin portion of Tn7.

Transformation of Tobacco

Leaf discs of *Nicotiana tabacum* cv Xanthi-nc were transformed with Agrobacterium cultures, containing the deletion constructs, by the leaf disc cocultivation method (Horsch et al., 1985). Transgenic plant lines were named for the deletion used (for example, a −903 PR-1a–GUS transformant was called −903-A). Approximately 15 independent transformants of each deletion were selected. Plants were rooted on medium containing no hormones and 100 μg/mL kanamycin. At approximately the five-leaf stage, each plantlet was vegetatively split and five identical plants were individually propagated. One of these was allowed to set seed and the remaining four were used for GUS assays following treatment with either water, SA, Carborundum, or tobacco mosaic virus (TMV). The water treatment was taken as an indication of the basal level expression from the promoter. Some T_1 plants were selfed, and the seeds were plated on kanamycin for germination. The resulting plants were selfed again and seed lines homozygous for kanamycin resistance were used for experiments involving T_3 plants.

Plant Treatments for Fluorometric GUS Assays

When transgenic plants were ~40 cm high, three leaves per plant were treated with the U1 strain of TMV, or mock inoculated with Carborundum and buffer (10 mM $NaPO_4$, pH 7), as previously described (Payne et al., 1990). Inoculated leaves were harvested 7 days later. For experiments with phytohormones, water, 50 mM salicylate, 1 mg/mL kinetin, 1 mg/mL 6-benzylaminopurine (BAP), and 1 mg/mL 1-naphthaleneacetic acid (NAA) (all from Sigma) were all applied to three leaves per plant using a paint brush. These leaves were harvested 2 days following treatment.

GUS Assays

Fluorometric GUS assays were performed essentially according to the method of Jefferson et al. (1987). Reactions were carried out in the presence and absence of 2 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) using 20 μL of supernatant from each sample (total reaction volume = 75 μL). Reactions were terminated after 60 min by the addition of 225 μL of 0.2 M Na_2CO_3. Duplicate fluorescence values for each sample were averaged, and the background fluorescence (reaction without MUG) was subtracted to obtain a concentration of methylumbelliferone for each sample.

BCA protein assays were performed on each sample according to the manufacturer's instructions (Pierce, Rockford, IL). Specific activities were calculated as nanomoles per milligram of protein per minute and converted to activity units (picomoles of methylumbelliferone per milligram of protein per minute). A value of 0.1 activity unit represents the lower detection limit of the assay indicating that samples with this level of activity were just detectable in an 18-hr assay.

For the in situ assays, tissue sections were cut by hand and placed overnight in 0.5 mg/mL X-gluc (5-bromo-4-chloro-3-indolyl glucuronide), 100 mM $NaPO_4$, pH 7, at 37°C (Jefferson et al., 1987). Some sections were cleared in 100% ethanol at room temperature.

Primer Extension Mapping

Primer extension reactions were carried out on total RNA, as described by Métraux et al. (1989), using a PR-1a–specific oligonucleotide with the following sequence: 5′-ATAGTCTTGTTGAGAGTTT-3′. Amounts of transcript generated for each sample were quantitated by detecting β-decay of phosphorus-32 with a Betascope 603 blot analyzer (Betagen, Waltham, MA).

RNA Extraction and Gel Blot Hybridization

RNA was purified from frozen tissue samples by phenol/chloroform extraction followed by lithium chloride precipitation (Lagrimini et al., 1987). Ten-microgram samples of total RNA were separated by electrophoresis through formaldehyde agarose gels and blotted to nylon membrane (GeneScreen Plus; New England Nuclear, Boston, MA), as previously described (Ausubel et al., 1987). Ethidium bromide was included in the sample loading buffer at 40 mg/mL, which allowed photography under UV light after electrophoresis to confirm equal sample loading. Probes were made by the random-priming method (Feinberg and Vogelstein, 1983) with a Prime Time C kit (International Biotechnologies, Inc., New Haven, CT). Hybridizations and washing were performed according to the method of Church and Gilbert (1984). Relative amounts of each transcript were quantitated with a Betascope, as described above.

Cycloheximide Treatments

CHX (Sigma), SA, H_2O, or SA plus CHX was injected by pricking a fully expanded tobacco leaf with a needle and forcing a small amount of liquid into the leaf with a 10-mL syringe. The water soaked area was marked and the plants were kept in the laboratory for 24 hr before harvesting the injected area. Leaves treated with SA plus CHX were pretreated with CHX alone for 30 min prior to the SA plus CHX treatment. Protein synthesis was determined by ^{35}S-methionine incorporation into trichloroacetic acid–precipitable material. The leaf tissue was injected with ^{35}S-methionine (Amersham, Arlington Heights, IL; 100 μCi/mL in distilled water) and after 1 hr the tissue was frozen in liquid nitrogen. The frozen tissue was ground in 2 × SDS sample buffer (Laemmli, 1970), and trichloroacetic acid precipitable counts were determined as described (Promega manual, "Translation in Vitro"; Promega, Madison, WI).

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