

Immediate Early Transcription Activation by Salicylic Acid via the Cauliflower Mosaic Virus *as-1* Element

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Transgenic tobacco plants carrying a number of regulatory sequences derived from the cauliflower mosaic virus 35S promoter were tested for their response to treatment with salicylic acid (SA), an endogenous signal involved in plant defense responses. β -Glucuronidase (*GUS*) gene fusions with the full-length (–343 to +8) 35S promoter or the –90 truncation were found to be induced by SA. Time course experiments revealed that, in the continuous presence of SA, the –90 promoter construct (–90 35S–*GUS*) displayed rapid and transient induction kinetics, with maximum RNA levels at 1 to 4 hr, which declined to low levels by 24 hr. Induction was still apparent in the presence of the protein synthesis inhibitor cycloheximide (CHX). Moreover, mRNA levels continued to accumulate over 24 hr rather than to decline. By contrast, mRNA from the endogenous pathogenesis-related protein-1a (PR-1a) gene began to accumulate at later times during SA treatment and steadily increased through 24 hr; transcription of this gene was almost completely blocked by the presence of CHX. Further dissection of the region from –90 and –46 of the 35S promoter revealed that the SA-responsive element corresponds to the previously characterized activation sequence-1 (*as-1*). These results represent a definitive analysis of immediate early responses to SA, relative to the late induction of PR genes, and potentially elucidate the early events of SA signal transduction during the plant defense response.

INTRODUCTION

Salicylic acid (SA) is an important mediator of the plant defense response to pathogens, yet only limited information exists on the nature of the SA signal transduction pathway. The first evidence for the role of SA in the induction of defense responses resulted from the application of a solution of aspirin (acetylsalicylic acid) to tobacco leaves (White, 1979). The plants were found to have enhanced resistance to tobacco mosaic virus (TMV), manifested by the reduced number and size of necrotic lesions. This resistance phenotype is similar to the phenomenon of systemic acquired resistance (SAR), which is characterized by a nonspecific “immunity” throughout the plant against a broad range of pathogens following initial infection by a necrotizing pathogen. Subsequently, it has been shown that TMV infection of tobacco of the resistant N genotype (e.g., *Xanthi-nc*) caused the accumulation of endogenous SA (Malamy et al., 1990, 1992; Yalpani et al., 1991). In these experiments, levels of SA in infected leaves began to accumulate at 24 hr postinfection and continued to rise for up to 8 days or more. In pathogen-infected cucumber, SA was identified in the phloem, and levels were found to increase coincident with the onset of SAR (Métraux et al., 1990; Rasmussen et al., 1991).

Increases in salicylate levels have also been correlated to changes in gene expression. It has been well established that during infection by pathogens, a battery of proteins are induced, including the pathogenesis-related (PR) protein families, which are thought to play an active role in the defense process (reviewed in Enyedi et al., 1992; Malamy and Klessig, 1992; Raskin, 1992a, 1992b; Yalpani and Raskin, 1993). In TMV-infected tobacco, mRNA from the PR-1 gene was first apparent at 36 hr postinfection, a few hours after SA levels began to rise, and increased steadily between 48 and 72 hr (Malamy et al., 1990). Ward et al. (1991) reported that exogenous SA can coordinately induce the transcription of nine classes of genes that are also induced by TMV. In these experiments, treatment of tobacco leaves with 50 mM SA produced an induction of PR genes that was detectable in 2 to 4 hr, increasing to high levels over the 48-hr test period. In another report, levels of PR-1 protein were proportional to the concentration of SA detected in exudates following treatment with exogenous SA or during SA accumulation following TMV inoculation (Yalpani et al., 1991).

Other evidence for salicylate involvement in defense responses comes from studies involving perturbations of SA levels. Incubation of infected *Xanthi-nc* tobacco above 28°C is known to disrupt lesion formation and the induction of PR genes (van Loon, 1975). Examination of the SA levels in these plants revealed that SA fails to accumulate at the elevated temperature (Yalpani et al., 1991; Malamy et al., 1992). Constitutive

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expression of PR proteins and strong resistance to several pathogens have been observed in the hybrid cross between *Nicotiana glutinosa* (resistant N genotype) and *N. debneyi* (susceptible genotype) (Ahl-Goy et al., 1992), and recently it has been determined that the hybrid plants contain levels of free SA 30 times higher than healthy Xanthi-nc tobacco (Yalpani et al., 1993). The opposite situation occurs in transgenic tobacco carrying the bacterial gene for salicylate hydroxylase (*nahG*), which converts SA to catechol (Gaffney et al., 1993). These plants fail to accumulate SA and do not induce acquired resistance to TMV.

Investigations into the components involved in the SA response have led to the characterization of an SA binding protein, which was identified as catalase, an H₂O₂-degrading enzyme (Chen and Klessig, 1991; Chen et al., 1993a, 1993b). This protein showed specificity for binding SA, which led to a reduction of enzymatic activity. By 3 hr after treatment with 1 mM SA, the amount of H₂O₂ in tobacco leaves had increased, and levels continued to increase over the 24-hr test period. Direct treatment of leaves with hydrogen peroxide induced PR-1a gene expression, raising the possibility that the pathway from pathogen infection to SA accumulation to PR gene induction may also involve the signal of peroxide accumulation. Whether the signals of increased SA and increased H₂O₂ are part of a single linear pathway in the defense response or have pleiotropic effects is currently unclear; it is also not clear whether additional targets for SA exist in the cell.

Other approaches to understanding the SA response have focused on the examination of promoter sequences that confer TMV and SA inducibility on the PR-1 and PR-2 genes (Ohshima et al., 1990; Hennig et al., 1993a; Uknes et al., 1993; van de Rhee and Bol, 1993; van de Rhee et al., 1993). Although the deletion analyses in these studies have narrowed down which regions of the promoters are involved in these responses, they have not yet pinpointed specific *cis* elements or any transcription factors that mediate the SA response. In all cases, however, SA-inducible constructs were similarly induced by TMV, consistent with a role for SA in mediating the nuclear activation of the TMV-induced defense response pathway.

These experiments clearly delineate a role for SA in the pathway from pathogen infection to the induction of PR genes and to acquired resistance, and in part, they have provided some information on how these events are mediated. Yet gaps in our understanding still exist. Many of the markers of the defense responses, such as increases in SA, induction of PR genes, and formation of lesions, are not detectable for many hours or even days. The events that occur during this time are almost completely unknown. For example, induction of PR-1 gene expression by SA is blocked in the presence of a protein synthesis inhibitor (Uknes et al., 1993; this study), suggesting that the synthesis of some unknown protein involved in PR-1 induction must first occur.

We have now characterized a novel SA-inducible element from the cauliflower mosaic virus (CaMV) 35S promoter as part of our continuing investigation of the transcriptional regulation of this prototypical plant promoter. The striking feature of

the SA response conferred by this sequence element is that induction was rapid, producing elevated mRNA levels within 30 min of treatment. Induction was also transient, with the amount of mRNA dropping to low levels by 24 hr. Further analysis of this SA-responsive element has identified it as the previously characterized activation sequence-1 (*as-1*), a binding site for a transcription factor of the basic leucine zipper family.

RESULTS

Induction of the CaMV 35S Promoter by SA Treatment

Experiments were performed using a number of transgenic plants carrying various CaMV 35S promoter and promoter subdomain- β -glucuronidase (*GUS*) fusions that were prepared in the course of this work or that have been described in our previous studies (Benfey and Chua, 1990; Benfey et al., 1990a, 1990b). Initially, leaves were treated with a variety of plant regulatory compounds to determine their influence on viral promoter activity. Responsiveness was assessed by histochemical staining and fluorometric quantitation of *GUS* activity (data not shown) (Jefferson et al., 1987). Induction by SA was observed, and among the constructs tested, the -90 35S-*GUS* fusion was found to be highly and consistently induced by SA in six independent transgenic lines. The full promoter construct (-343 35S-*GUS*) had a high expression level in the absence of SA but also showed a further induction by SA treatment (data not shown). None of the other subdomain constructs appeared to be inducible. Analysis of the effects of other plant compounds was not pursued.

As shown in Figure 1, three test constructs were analyzed further by treatment with water or SA, followed by quantitative S1 nuclease protection (Fluhr et al., 1986). In agreement with the histochemical data, the -343-*GUS* construct showed a high constitutive level of activity that increased modestly following SA treatment (Figure 1A, -343 35S). A truncation of the promoter to -90 showed reduced activity overall; however, treatment with SA resulted in a marked increase in the steady state RNA level (Figure 1A, -90 35S). The construct denoted B-46 35S-*GUS*, which contains the -343 to +8 sequence lacking the -90 to -46 region, was not induced by SA (Figure 1A, B-46 35S). As in our previous studies (Benfey et al., 1989), each construct also contains the -941 35S-chloramphenicol acetyltransferase (*CAT*) fusion (Figure 1B) for use as a reference gene. The -941-*CAT* construct, carrying some of the same sequence information as the test constructs, displayed some induction by SA on top of the high constitutive level of expression. It should be noted that the S1 nuclease protection conditions were not optimized to detect the -941-*CAT* fusion, and its RNA level could be underestimated (see Methods). We concluded from these results that the -90 35S promoter is clearly induced by SA and that induction arises via a regulatory element located between -90 and -46 bp.

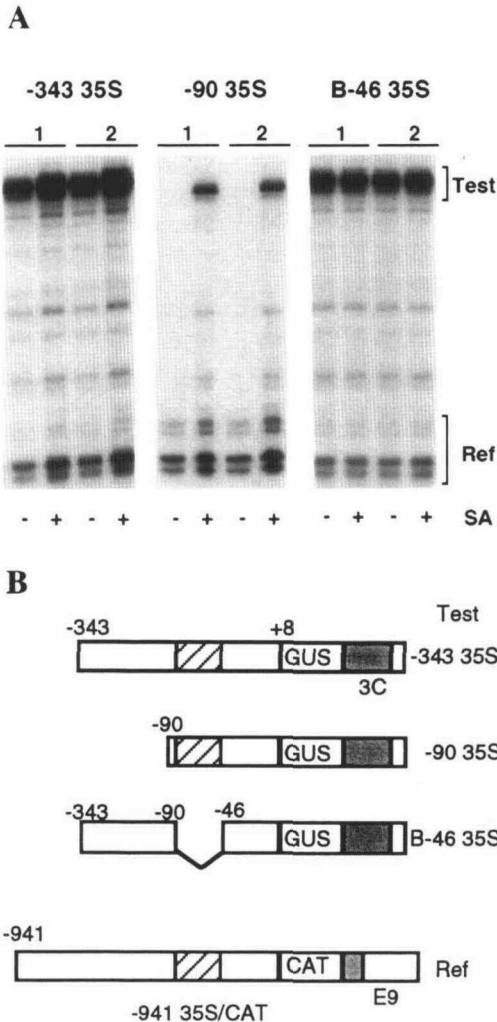


Figure 1. Induction of 35S-GUS fusions by SA Treatment.

(A) Leaves treated with H₂O (-) or 2 mM SA (+) for 2 hr. Results of quantitative S1 nuclease protection using 20 μg of total RNA from two independent transgenic plants (1 and 2) of each construct are shown. Signal denoted "test" is the S1 protection signal derived from the 35S-GUS fusion transcript; "Ref" is the signal derived from the -941 35S-CAT fusion transcript (see Methods).

(B) Promoter fusion constructs. The -343-GUS, -90-GUS, and B-46-GUS constructs are designated as the test promoter fusions, and -941-CAT is designated as the reference promoter fusion. The hatched boxes in the promoter regions contain the *as-1 cis* element. The shaded portions in pea *rbcS-3C* (3C) and *rbcS-E9* (E9) 3' fragments represent the sequences that give rise to the S1 nuclease protection signals.

Rapid, Transient, and Concentration-Dependent Induction of the -90 35S Promoter

The results of experiments testing the SA dose response of the -90 promoter are shown in Figure 2A. It was found that,

at the concentrations tested, induction was detectable at 2 μM, with increasingly greater levels of mRNA at 20 and 200 μM, and was saturated between 200 μM and 2 mM SA (Figure 2A). An investigation of the induction kinetics of the -90 35S promoter is shown in Figure 2B. In untreated leaves (Figure 2B, lane 1) or leaves treated with H₂O for up to 24 hr (lanes 2 to 6), no transcription from the -90 promoter was detectable.

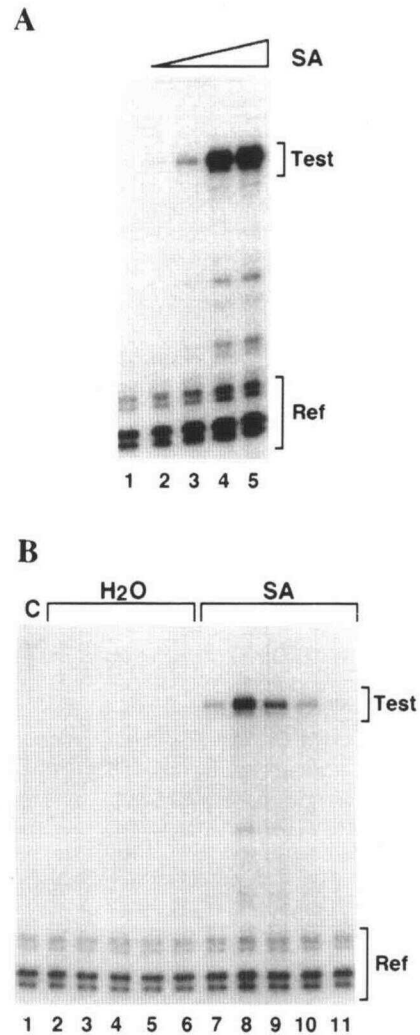


Figure 2. SA Responsiveness of the -90-35S Promoter.

(A) Concentration dose response. SA concentrations are lane 1, 0; lane 2, 2 μM; lane 3, 20 μM; lane 4, 200 μM; lane 5, 2 mM. Incubations were conducted for 2 hr. Total RNA (30 μg per sample) was used for S1 nuclease protection.

(B) Induction kinetics. Lane 1, untreated control leaves (C); lanes 2 to 6, leaves treated with H₂O for 0.5, 1, 4, 8, and 24 hr, respectively; lanes 7 to 11, treated with 2 mM SA for 0.5, 1, 4, 8, and 24 hr, respectively. Total RNA (25 μg per sample) was used. The notations "Test" and "Ref" are the same as given in Figure 1.

However, in leaves maintained in the continuous presence of 2 mM SA (lanes 7 to 11), transcription was rapidly and transiently induced. The *GUS* mRNA level was readily detected at the first time point (0.5 hr), reached a peak by 1 to 2 hr, and slowly declined to background levels by 24 hr. Phosphorimaging analysis (Molecular Dynamics, Sunnyvale, CA) of RNA gel blots revealed that the induction at the peak time point (2 hr) was at least 20 times over the basal level (data not shown). Expression of the reference gene was fairly constant in these experiments, except for slight increases presumably arising from SA induction.

Effect of Cycloheximide on SA-Induced Gene Activation

The rapidity of the response to SA suggested that induction may occur via preexisting molecules in the cell. To test whether induction requires new protein synthesis, time course experiments were repeated in the presence of the protein synthesis inhibitor cycloheximide (CHX) and examined by RNA blot analysis. The results are shown in Figure 3. As before, -90 -*GUS* transcription was detectable neither in untreated leaves (Figure 3A, lane 1) nor in leaves treated with water over a 24-hr period (Figure 3A, lanes 2 to 6). Treatment with 2 mM SA resulted in an induction profile similar to that in Figure 2B, peaking at 2 to 4 hr and decreasing to low levels by 24 hr (Figure

3A, lanes 7 to 11). When leaves were preincubated for 1 hr in the presence of 20 μ g/mL CHX and then transferred to a solution containing both 2 mM SA and 20 μ g/mL CHX, induction occurred as rapidly as in the absence of CHX; however, the transient kinetics changed. Rather than declining at later time points, the *GUS* mRNA continued to accumulate during the test period of 24 hr (Figure 3A, lanes 17 to 21). Treatment of leaves with CHX alone resulted in a small but detectable increase in the basal level of -90 -*GUS* transcription (Figure 3A, lanes 12 to 16).

As an endogenous control, we probed the same RNA blot for PR-1a mRNA. PR-1a is one of the best characterized defense-related genes expressed during SAR, and it is also known to be highly inducible by exogenous SA (for reviews, see Enyedi et al., 1992; Malamy and Klessig, 1992; Raskin, 1992a). In our experiments, the PR-1a gene was induced to high levels by SA (Figure 3A, lanes 7 to 11). However, in contrast to the -90 -*GUS* gene fusion, the activation of PR-1a was not evident until 4 hr of treatment and continued to increase to higher levels over 24 hr. More significantly, in tissue that had been treated with both SA and CHX, the induction of the PR-1a gene was essentially blocked (Figure 3A, lanes 17 to 21). Unlike the -90 -*GUS* construct, CHX treatment alone did not appear to affect the basal transcript level of the PR-1a gene during the 24-hr time course (Figure 3A, lanes 12 to 16). Hybridization of the blot with a control probe (β -ATPase) demonstrated even loading of all lanes. These results indicated that

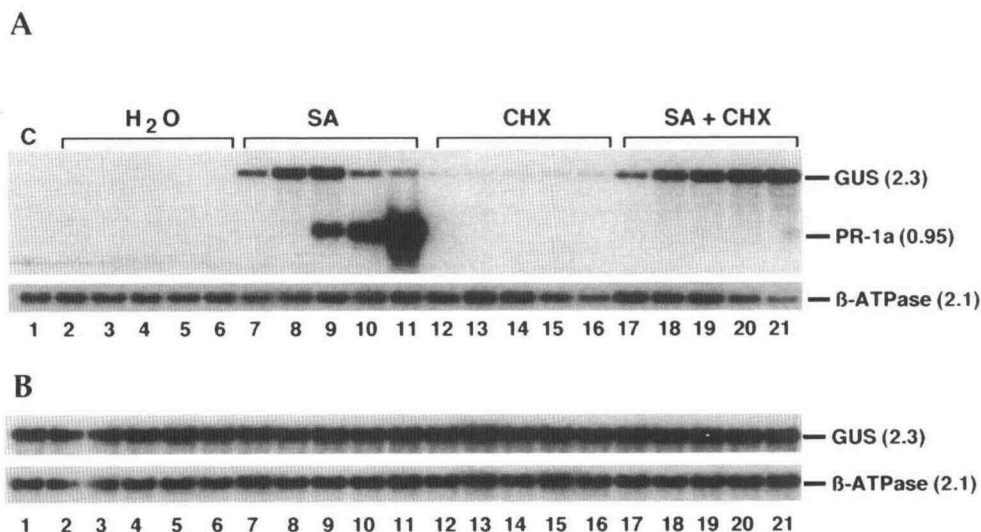


Figure 3. Effects of CHX on SA-Induced Gene Activation.

(A) -90 -*GUS* transgenic plants.

(B) B-46-*GUS* transgenic plants.

Lanes 1, untreated control leaves (C); lanes 2 to 6, treated with H₂O; lanes 7 to 11, treated with 2 mM SA; lanes 12 to 16, treated with 20 μ g/mL CHX; lanes 17 to 21, treated with 20 μ g/mL CHX and 2 mM SA for 0.5, 1, 4, 8, and 24 hr, respectively (see Methods). The RNA blots (30 μ g total RNA per lane) were sequentially hybridized with *GUS*, PR-1a (in [A] only), and β -ATPase probes. The corresponding hybridization signals are indicated on the right.

induction of the -90 35S-*GUS* construct and the PR-1a gene occurs via different mechanisms that can be distinguished by the requirement for protein synthesis.

To determine if the transient nature of the SA-induced accumulation of the *GUS* mRNA was a consequence of changes in the promoter activity versus changes in the mRNA stability, this set of experiments was also performed with plants carrying the B-46 35S-*GUS* fusion construct, which differs from the -90 -*GUS* construct only in promoter sequences (refer to Figure 1B). As shown in Figure 3B, the level of *GUS* mRNA transcribed from the B-46 promoter remained essentially constant in each time course, regardless of chemical treatment. A similar result was also observed for the β -ATPase gene. Because no differences exist between the transcripts produced from the -90 -*GUS* and B-46-*GUS* constructs, it is unlikely that changes in mRNA stability are responsible for the selective decline of *GUS* mRNA from the -90 promoter at late time points following SA treatment. Rather, this behavior must reflect the transcriptional activity of the promoter per se.

A 21-bp *as-1* Element Is Sufficient to Confer the SA Response

Earlier efforts from our laboratory to investigate the activity of the CaMV 35S promoter led to the definition of several *cis* elements, including one in the region of -83 to -63 designated *as-1* (Lam et al., 1989). The *as-1* element was shown to be the primary determinant for the transcriptional activity of the -90 35S promoter (Benfey et al., 1989; Fang et al., 1989; Lam et al., 1989). Furthermore, nuclear extracts have been shown to contain a sequence-specific *as-1* binding activity, designated ASF-1 (Lam et al., 1989). To test whether the *as-1* element was involved in SA induction, we made synthetic oligonucleotides containing either a single or four tandem copies of a 21-bp wild-type or mutant *as-1* sequence and inserted these upstream of a minimal TATA promoter-*GUS* fusion (X-*GUS*-46; Benfey et al., 1990a). The sequences of these constructs are given in Figure 4B. Mutant oligonucleotide sequences contained base substitutions that had been previously shown to impair *as-1* activity (Lam et al., 1989). Transgenic plants containing these constructs were examined for their ability to respond to a 2-hr treatment of water, 2 mM SA, or 2 mM SA and 20 μ g/mL CHX. As shown in Figure 4A, leaves from plants containing a single copy of the *as-1* sequence (A1-46) displayed SA inducibility at a level comparable to the -90 promoter regardless of the presence of CHX. However, plants containing the mutated *as-1* site (A1m-46) failed to be induced by SA or the combination of SA and CHX. In plants containing four copies of *as-1* (4A1-46), both an enhanced basal activity and an SA-induced activation were evident, whereas no promoter activity was detected in 4A1m-46 plants. The -941 35S-*CAT* reference transcript was detectable in all plants, and, as before, slight elevation of reference transcript levels was detected following SA treatment. These experiments demonstrate that the wild-type 21-bp *as-1* element retains the same level of SA-inducibility as the intact -90 promoter construct

and that this activity requires specific bases in the core sequences of the *as-1* element.

To test whether the *as-1* element can confer SA inducibility on a heterologous promoter, we tested constructs in which the wild-type or the mutant *as-1* sequence was created in the -109 to -89 region of the ribulose biphosphate carboxylase small subunit 3A (*rbcS-3A*) promoter by specific nucleotide substitutions, as shown in Figure 5B (Lam et al., 1989). *rbcS-3A* is a photosynthetic gene and is not SA inducible (data not shown). The results are shown in Figure 5A. When leaf sections from light-grown A1-*rbcS-3A* transgenic plants were treated with water only, a high basal level of expression was observed (Figure 5A, lane 1). Treatment with 2 mM SA considerably enhanced the level of mRNA from this promoter construct, regardless of the presence of CHX (lanes 2 and 3). In contrast, no SA induction was observed in plants containing the A1m-*rbcS-3A* construct (Figure 5A). This gain-of-function for the *rbcS-3A* promoter demonstrated that the 21-bp *as-1* element is sufficient to act as a portable SA response element.

SA Induction via Homologous Sequence Elements

Sequence elements with homology to the *as-1* site are found in the promoters of a number of T-DNA opine synthase genes (Bouchez et al., 1989; Fromm et al., 1989; Lam et al., 1990). Previously, gel shift analysis has demonstrated that ASF-1 can also bind to these sequence elements, but not to corresponding sequences with core base substitutions (Fromm et al., 1989; Lam et al., 1990). To determine if these sequences are also able to confer SA responsiveness, we tested two of these elements, namely *ocs* and *nos* from the octopine and nopaline synthase gene promoters, respectively, as *GUS* fusions in transgenic plants. Our results are shown in Figure 6. Plants carrying constructs with either a single copy of *ocs* or four copies of *nos* were found to be SA inducible in the presence and absence of CHX. The copy number of the sequence element appeared to influence the degree of induction, as observed with the *as-1* constructs in Figure 4. However, without the complementary constructs (four copies of *ocs* and one copy of *nos*), it is not definitive. Base substitutions in either the core sequence of the *ocs* or *nos* elements diminished the SA response. Thus, SA-induced transcription results correlate with ASF-1 DNA binding activity in vitro.

To further analyze the extent to which transcription activity in vivo correlated with binding specificity of ASF-1 in vitro, we tested synthetic -90 promoter constructs in which the native *as-1* elements were replaced by either the palindromic consensus sequences P1 or P2 (Figure 6B). In gel shift experiments, ASF-1 showed site-specific binding to both synthetic sites with higher affinity than for the native *as-1* site and with the greatest affinity for the P1 consensus elements (X.-F. Qin and N.-H. Chua, unpublished results). Both palindromic sites also resulted in SA-induced transcription (Figure 6A), with greater overall levels from the P1 construct. Therefore, all constructs tested in this study showed SA-induced transcription behavior that correlated with ASF-1 binding activity in vitro.

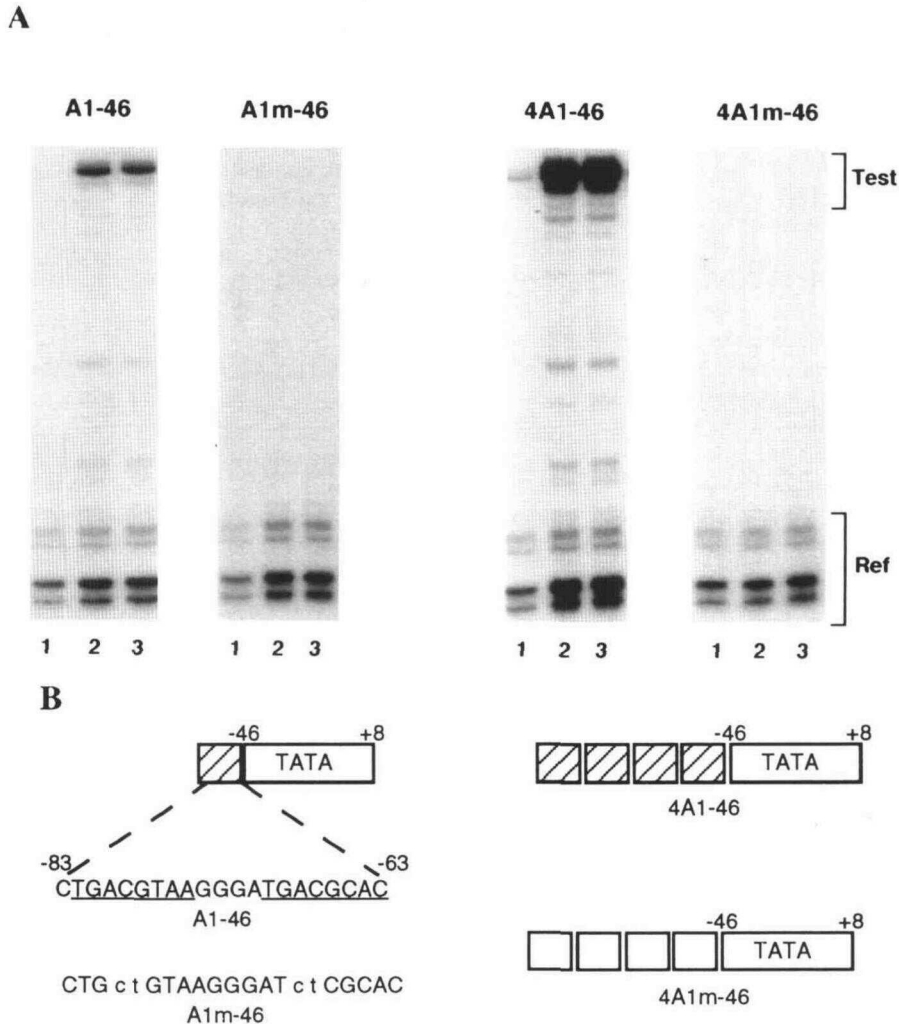


Figure 4. A 21-bp *as-1* Element Is Sufficient To Confer SA Responsiveness.

S1 analysis of A1-46, A1m-46, 4A1-46, and 4A1m-46 transgenic plants following SA treatment.

(A) Lanes 1, leaves treated with water; lanes 2, treated with 2 mM SA; lanes 3, treated with 20 μ g/mL CHX plus 2 mM SA for 2 hr. Total RNA (30 μ g per sample) was used for the S1 nuclease protection. The notations "Test" and "Ref" are the same as given in Figure 1.

(B) The test promoter constructs and the sequences of the *cis* elements are illustrated. Wild-type *as-1* elements are represented by hatched boxes, and mutant elements are represented by open boxes. The numbers above the sequence indicate the positions in the native promoter. The ASF-1 binding sites are underlined, and base substitutions in the mutant element are in lowercase letters.

DISCUSSION

Through the investigation of the responses of a plant viral promoter, we have serendipitously revealed a *cis* element that can confer SA inducibility with kinetics distinct from previously characterized SA-responsive genes (e.g., PR-1). Because several environmental factors, such as temperature, hormones, and nutrition, can influence viral growth in animal cells, we tested whether the CaMV 35S promoter was also sensitive to

environmental cues in plants. Our initial efforts revealed that transgenic plants carrying *GUS* reporter gene constructs transcribed from 35S promoter sequences displayed SA-inducible *GUS* activity. Low-level SA inducibility was conferred by the -941 to +8 region of the 35S promoter and by -343 to +8, whereas greater induction was conferred by truncation to -90. Consistent with previous studies, the -90 promoter construct was expressed at low level in leaves (Benfey et al., 1989); therefore, induction by SA was more apparent than with longer constructs that displayed higher expression levels overall,

presumably as a result of the combination of other non-SA responsive *cis* elements present in those promoters. Induction of GUS enzyme activity was approximately fivefold; however, at the mRNA level, induction of -90-GUS has been found to be ~20-fold over basal levels. A similar level of SA-induced GUS activity was observed by van de Rhee et al. (1993) by using a -90-GUS construct as a minimal promoter cassette to analyze PR-1 promoter sequences. In this study, the minimal promoter by itself or in combination with some PR-1 sequences consistently showed an SA-specific induction of approximately threefold, whereas other PR-1--90-GUS constructs showed higher levels of induction. Although van de Rhee et al. were cognizant of SA induction arising from the -90 sequences, the inadvertent combination of sequences conferring SA responsiveness from an immediate early gene with those from a late gene obfuscates the actual SA responses of the PR-1 gene.

Sensitivity of the -90-GUS construct was determined by treating tobacco leaves with a range of SA concentrations. The threshold for induction was ~2 μM SA, and the half-maximal inducing concentration was between 20 and 200 μM SA. Hennig et al. (1993b) reported that induction of the PR-1a gene had a threshold of 10 to 100 μM SA injected into tobacco leaves, and greater induction was evident with 500 μM and 1 mM injected SA. Yalpani et al. (1991) detected the presence of the PR-1a protein by feeding excised tobacco leaves with at least 29 μM SA. PR-1a protein levels rose steadily with feeding up to 72 μM SA. Saturation was not demonstrated in either of these studies, so it is unclear what the half-maximal effective concentration of SA may be for PR-1a induction. Phytotoxicity of SA in the millimolar range may be a barrier to such determinations. UDP-glucose:SA glucosyltransferase is an SA-inducible enzymatic activity in oat roots (Yalpani et al., 1992a, 1992b). Induction of this activity has a threshold of ~10 μM SA and a half-maximal effective concentration of ~100 μM. Despite the differences in methods and materials in these studies, all systems, including -90-GUS, respond to SA in the micromolar range.

Kinetic analysis of -90-GUS transcription following SA treatment demonstrated that induction was rapid. By 30 min, an increase in transcript level was apparent. In comparison with the induction of the PR-1a gene, the entire transient activation of -90-GUS was essentially completed by the time PR-1a mRNA began to accumulate (Figure 3A). Pretreatment of plants with CHX did not block SA-induced transcription from the -90 promoter; rather, this combination of treatments disrupted the down-regulation after 4 hr, resulting in a hyperaccumulation of mRNA. Because CHX treatment alone produced an elevated basal level of transcription, one possibility is that a labile protein factor functions in the negative regulation of this promoter. Uknes et al. (1993) have also reported CHX disruption of PR-1a expression. They further noted that low levels of CHX stimulated expression of PR-1a, perhaps as a stress response of the plant to partial suppression of protein synthesis potentially brought about by virus infection. Others have observed transcriptional induction by CHX at concentrations too low

to disrupt protein synthesis (Mahadevan and Edwards, 1991; Rao and Mufson, 1993). This result has been interpreted as evidence for a direct induction mechanism involving protein phosphorylation.

One 35S promoter construct that failed to be induced by SA is B-46, which contains the upstream sequence of -343 to +8 but has the internal deletion of -90 to -46. This region encompasses the previously characterized *as-1* element at -83 to -63 (Lam et al., 1989). By testing transgenic plants

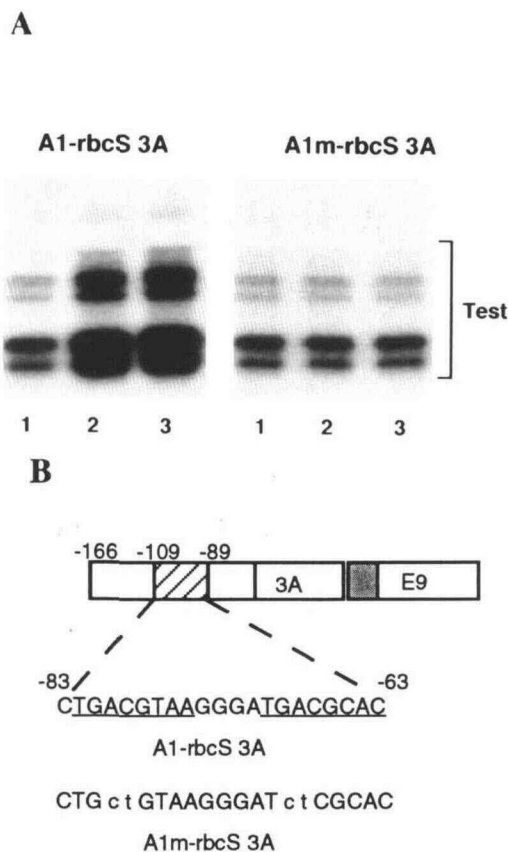


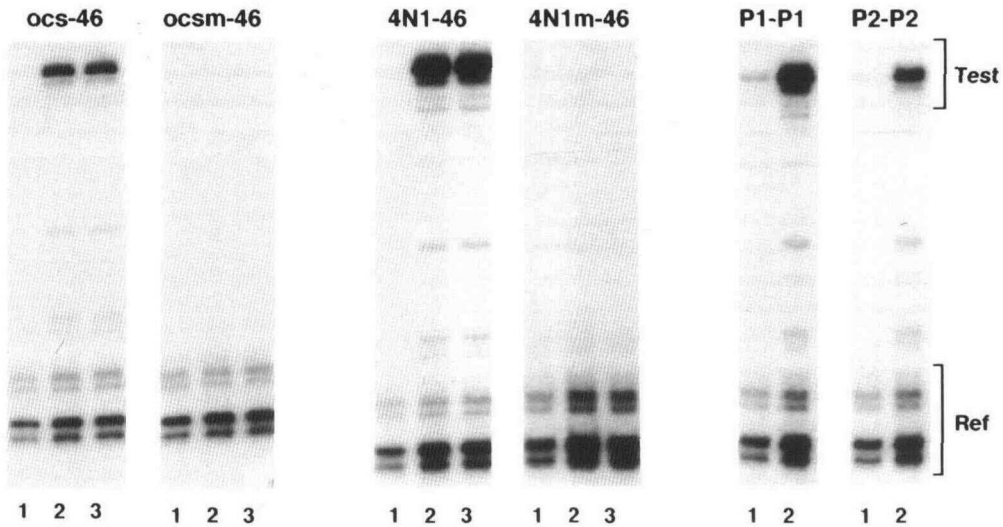
Figure 5. *as-1* Confers SA Inducibility on the *rbcS-3A* Promoter.

S1 analysis of A1-*rbcS-3A* and A1m-*rbcS-3A* transgenic plants following SA treatment.

(A) Lanes 1, leaves treated with water; lanes 2, treated with 2 mM SA; lanes 3, treated with 20 μg/mL CHX plus 2 mM SA for 2 hr. Total RNA (30 μg per sample) was used for the S1 nuclease protection. The A1-*rbcS-3A* and A1m-*rbcS-3A* transgenic plants did not contain the -941 35S-CAT reference promoter fusion; therefore, only one S1 signal ("Test") derived from the E9 3' sequence was detected (see Methods).

(B) The test promoter constructs and the sequences of the *cis* elements are illustrated. The *as-1* element is represented by a hatched box. The numbers above the sequence and boxes indicate the positions in the native promoters. The ASF-1 binding sites are underlined and base substitutions in the mutant element are in lowercase letters. The shaded box indicates the region giving rise to the S1 protection signal.

A



B

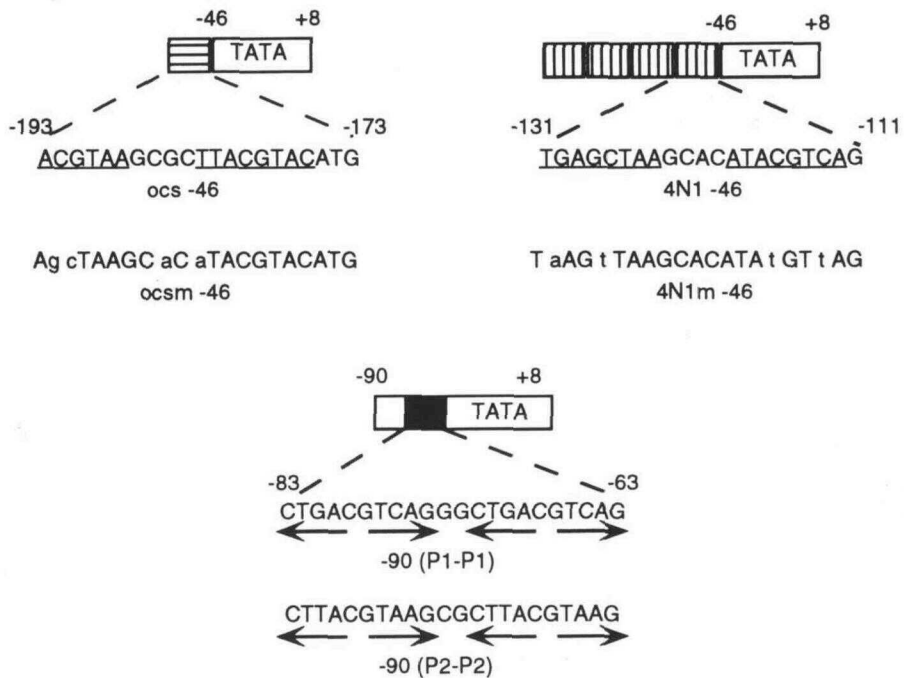


Figure 6. Sequence Elements Related to *as-1* also Confer SA-Inducible Transcription.

(A) S1 analysis of *ocs-46*, *ocsm-46*, *4N1-46*, *4N1m-46*, $-90(P1-P1)$, and $-90(P2-P2)$ transgenic plants following SA treatment. Lanes 1, leaves treated with water; lanes 2, treated with 2 mM SA; lanes 3, treated with 20 $\mu\text{g}/\text{mL}$ CHX plus 2 mM SA for 2 hr. Total RNA (30 μg per sample) was used for the S1 nuclease protection. The notations "Test" and "Ref" are the same as given in Figure 1.

(B) Test promoter constructs and the sequences of the *cis* elements are illustrated. The *ocs* element is represented by the horizontally striped box, the *nos* elements are represented by vertically striped boxes, and the synthetic palindromic elements are represented by the solid box. The numbers above the sequences and boxes indicate the positions in the native promoters. The ASF-1 binding sites are underlined, and base substitutions in the mutant elements are in lowercase letters. Arrows highlight the symmetry of the palindromic elements. Constructs *ocs-46* and *ocsm-46* are also known as *21ocswt-46* and *21ocsmu-46* (Fromm et al., 1989).

with constructs consisting solely of the *as-1* sequence fused to -46-GUS, we were able to reproduce SA induction to the same levels as the intact -90 construct. The *as-1* element is composed of a pair of tandem motifs characterized by the half-site TGAC. Footprinting and gel shift analyses have demonstrated a binding activity from plant nuclear extracts associated with these sequences, denoted ASF-1 (Lam et al., 1989). A component of ASF-1 has been identified as the transcription activator TGA1a, a protein belonging to the basic leucine zipper family of transcription factors (Katagiri et al., 1989). Binding activity in vitro and transcriptional activity in vivo were shown to be sensitive to base substitution in the core sequences (Figure 4); thus, the interaction of ASF-1 with *as-1* correlates with the regulatory function of this *cis* element in vivo. In the current study, we also found that SA inducibility could be disrupted by the same base substitutions.

In addition to *as-1*, ASF-1 also binds to the closely related sequence elements in the *Agrobacterium ocs* and *nos* gene promoters (Fromm et al., 1989; Lam et al., 1990). Our current investigations have revealed that these two sequence elements can also confer SA inducibility and, like *as-1*, are attenuated if base substitutions are made in the core sequences. A recent paper has demonstrated SA induction of *nos* promoter-CAT fusions by measuring CAT enzyme activity (Kim et al., 1993). Deletion analysis of the *nos* promoter determined that the region spanning -130 to -112, containing the tandem *as-1*-like sequences (see Figure 6B), is necessary for the SA response; however, because no gain-of-function experiment was performed, it was not known whether the deleted sequence by itself could confer SA responsiveness on a heterologous promoter. Induction of CAT activity was fairly rapid following SA treatment, becoming evident between 2 and 4 hr and increasing thereafter. Although sensitivity to CHX was not tested, such kinetics may reflect immediate early induction of transcription, as demonstrated here for the -90 promoter construct. Unlike -90-GUS transgenic tobacco, the *nos*-CAT transgenic tobacco required ~0.5 mM SA or more for half-maximal activation, although the different experimental approaches used by Kim et al. and in our current study make a direct comparison difficult.

In addition to the *ocs* and *nos* elements, we have shown that synthetic consensus binding sites, designated P1 and P2, are also able to confer SA-induced transcription. Each site was designed to create a perfect palindrome based on each half-site of the element at -82 to -75. The higher level of transcription with the -90(P1-P1) promoter construct correlates well with a greater extent of ASF-1-DNA complex formation in electrophoretic mobility shift assays, relative to P2 and the native *as-1* elements (X.-F. Qin and N.-H. Chua, unpublished results). Thus, the transcriptional activity of all five promoter constructs tested for SA induction strongly correlates with ASF-1 binding activity in vitro, suggesting that SA induction is mediated by ASF-1 or a related factor.

The serendipitous observation that a plant cellular factor is involved in the response of viral and bacterial promoters to SA with immediate early kinetics was surprising. Although it

has not yet been demonstrated whether CaMV or *Agrobacterium* causes an increase of endogenous SA in tobacco, these pathogenic organisms may have been selected to subvert an early plant defense response to stimulate transcription of their own genes and thereby establish infection. Another possibility is that *as-1* simply serves to promote viral transcription in root cells where ASF-1 is most abundant (Fromm et al., 1989; Katagiri et al., 1989). Whereas the level of -90-GUS mRNA is higher in water-treated roots relative to leaves, this level increases markedly following SA treatment (X.-F. Qin and N.-H. Chua, unpublished results). Thus, SA further enhances transcription from the 35S promoter, which consists of several *cis* elements that combine to produce a high level of expression in a range of tissues (Benfey and Chua, 1990).

In consideration of the fact that ASF-1 is a plant factor, it is likely that endogenous plant genes exist that are transcriptionally activated by SA with immediate early kinetics by the same mechanism and latent factors that mediate the -90 promoter response, potentially as the initial steps in the establishment of SAR. Some of the most rapid changes associated with pathogen infection are changes in ion fluxes and the pattern of protein phosphorylation (Dietrich et al., 1990; Felix et al., 1991). It is possible that the rapid phosphorylation (or dephosphorylation) of latent factors leads to immediate early, CHX-insensitive transcriptional activation. Later desensitization would require a phosphatase (or kinase) to reverse the modification, and inhibition of the synthesis or activity of such a molecule by CHX would lead to the superinduction of RNA from the target gene.

In animal systems, a variety of cytokines are known to induce transient immediate early transcription of a number of genes by mechanisms that are independent of new protein synthesis and that can be superinduced by stimulation in the presence of protein synthesis inhibitors (Greenberg et al., 1986; Almendral et al., 1988; Zipfel et al., 1989). In several cases, including *c-fos*, somatostatin, and guanylate binding protein gene promoters, the transient activation of transcription has been correlated with a transient increase in phosphorylation of the ternary complex factor (TCF), cAMP responsive factor (CREB), and p91 transcription factor complexes, respectively (Hagiwara et al., 1992; Shuai et al., 1992; Zinck et al., 1993). The subsequent dephosphorylation of these factors correlates with the decline in promoter activity. A further finding is that induction in the presence of phosphatase inhibitors can produce a superinduction of transcription (Zinck et al., 1993). Further testing is necessary to establish if a similar cycle of phosphorylation of latent factors is indeed in operation in the immediate early response of plant cells to SA.

METHODS

Plasmid Construction and Transgenic Plants

The constructs A1-46 and A1m-46 were made by direct cloning of 21-bp complementary oligonucleotides (for sequences, see Figure 4),

with 4-bp HindIII and XhoI overhangs placed at the 5' and 3' ends into the X- β -glucuronidase (*GUS*)-46 vector (Benfey et al., 1990a, 1990b). The constructs 4A1-46 and 4A1m-46 were made by subcloning of the previously described wild-type and mutant activation sequence-1 (*as-1*) tetramer sequences (Lam et al., 1990) in the same vector. Sequences of all constructs were confirmed by sequencing.

Nicotiana tabacum cv SR1 was transformed by the standard method of Benfey et al. (1989). At least 10 independent transgenic lines for each construct were generated. Transgenic plants harboring -90, B-46, A1-ribulose biphosphate carboxylase small subunit (*rbcS*)-3A, A1m-*rbcS*-3A, octopine synthase (*ocs*--46; *ocsm*--46 (the latter two also known as 21ocswt-46 and 21ocsmu-46), 4N1-46, and 4N1m-46 promoter-*GUS* constructs were generated in our previous studies (Benfey et al., 1989; Fromm et al., 1989; Lam et al., 1989), and A1--46, A1m--46, 4A1--46, 4A1m--46, -90(P1-P1), and -90(P2-P2) constructs were generated in this work. Seeds were germinated on a medium containing Murashige and Skoog (1962) salts (JRH Biosciences, Lenexa, KS), 3% sucrose, 0.7% agar, 100 μ g/mL kanamycin, and 300 μ g/mL carbenicillin. Kanamycin-resistant seedlings were assayed for chloramphenicol acetyl transferase (CAT) activity (encoded by the -941 35S-CAT reference gene fusion). Only those with CAT activity were chosen for further experiments. The seedlings were either maintained on the same medium in Plantcons (ICN Biomedicals, Inc., Costa Mesa, CA) at 26°C under 16-hr-light/8-hr-dark cycle or transferred to soil and grown in pots in a greenhouse.

Chemical Treatment

Fully expanded leaves either from Plantcon cultures or potted plants were used and essentially the same results were obtained: For salicylic acid (SA) treatments, plant leaves were excised, midribs removed, and cut to \sim 2-cm² sections. Leaf sections were floated on a solution containing sodium salicylate, pH 6.8, at the indicated concentration. Distilled water was used as a control. For inhibition of protein synthesis, the plant tissues were first soaked in 20 μ g/mL cycloheximide (CHX) solution for 1 hr and then in solutions containing 20 μ g/mL CHX and 2 mM sodium salicylate or 20 μ g/mL CHX only, as control. All treatments were performed at room temperature and under constant illumination. In experiments with CHX, protein extracts were made and the *GUS* activity was measured by the fluorometric method (Jefferson et al., 1987). Typically, a 24-hr treatment with SA induced the *GUS* activity in the -90 35S-*GUS* transgenic plants to 5 to 10 times over the basal level. Pretreatment with 20 μ g/mL CHX reduced *GUS* activity to less than 5% (data not shown), which reflected the overall extent of inhibition of total protein synthesis.

RNA Analysis

Total RNA was isolated from pooled samples by a glass bead/RNAMATRIX method (Bio-101, La Jolla, CA) according to the manufacturer's protocol. Quantitative S1 nuclease protection was performed essentially as described by Fluhr et al. (1986). The 250-bp HindIII-ClaI fragment from the pea *rbcS*-3C gene 3' sequence was used as probe. Hybridization conditions were modified according to Ausubel et al. (1987) to increase specificity and sensitivity. The hybridization solution contained 80% formamide, 40 mM Pipes, pH 6.5, 400 mM NaCl, and 1 mM EDTA. Hybridization was performed at 37°C, except in experiments with RNA samples from A1-*rbcS*-3A and A1m-*rbcS*-3A constructs, which were hybridized at 32°C. Under these conditions,

the test gene fusion transcript (except for the A1-*rbcS*-3A and the A1m-*rbcS*-3A) gave a major protection fragment of 230 nucleotides in length, whereas the reference gene fusion transcript resulted in a cluster of bands of \sim 89 nucleotides. Because the optimal hybridization conditions are different for the test and reference genes, the absolute level of expression cannot be directly compared from the S1 protection signals. A GeneScreen membrane (New England Nuclear, Boston, MA) was used for RNA blot analysis. RNA transfer, membrane treatment, and hybridization were performed according to the manufacturer's protocol. A 2.0-kb XbaI-BamHI fragment of the *GUS* coding region (Jefferson et al., 1987), a 0.7-kb EcoRI fragment from the tobacco PR-1a gene CNT3 (Memelink et al., 1987), and a 1.3-kb XbaI-HindIII fragment from the *N. plumbaginifolia* mitochondrial β -ATPase gene *apt2-1* (Boutry and Chua, 1985) were used as hybridization probes. Filters were successively hybridized with the *GUS*, PR-1a, and β -ATPase probes after the previous one had been stripped off, except as shown in Figure 3A in which the *GUS* probe was not stripped prior to hybridization with PR-1a. At least four independently derived transgenic lines for each construct were analyzed and similar results were obtained. Figures were prepared by scanning films from RNA blot analyses and arranging images using Photoshop (Adobe Systems, Mountainview, CA) and Canvas (Deneba Systems, Miami, FL) software. Photographs were obtained using a Kodak XL-7700 dye-sublimation printer.

ACKNOWLEDGMENTS

We thank Drs. Philip N. Benfey, Eric Lam, Hillel Fromm, and Philip M. Gilmartin for transgenic seeds; Dr. Johan Memelink for the PR-1a clone CNT3; Dr. Alan Brunelle for advice on computer graphic work; and members of the Chua laboratory for helpful discussion. L.H. was supported by a National Institutes of Health Fogarty Postdoctoral Fellowship, and D.M.H. was supported by a National Science Foundation Plant Biology Fellowship. This work was supported by a grant from the Monsanto Company (St. Louis, MO).

Received February 15, 1994; accepted April 11, 1994.

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