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cis-Regulatory Elements Involved in Ultraviolet Light Regulation and Plant Defense

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An elicitor-regulated transient expression system was established in soybean protoplasts that allowed the identification of *cis*-regulatory elements involved in plant defense. The 5′ region of an ultraviolet (UV) light-inducible and elicitor-inducible *chs* gene (*chs*1) of soybean was subjected to deletion analysis with the help of chimeric *chs-nptII/gus* gene constructs. This analysis delimited the sequences necessary for elicitor inducibility to −175 and −134 of the *chs*1 promoter. The same soybean sequences were able to direct elicitor inducibility in parsley protoplasts, suggesting a conserved function of *cis*-acting elements involved in plant defense. In addition, this region of the soybean promoter also promotes UV light inducibility in parsley protoplasts. However, in contrast to the elicitor induction, correct regulation was not observed after UV light induction when sequences downstream of −75 were replaced by a heterologous minimal promoter. This result indicates that at least two *cis*-acting elements are involved in UV light induction.

INTRODUCTION

Flavonoids serve a variety of functions in plants and accumulate in response to different environmental stimuli including UV light irradiation, pathogen attack, and wounding (for recent review, see Dangl et al., 1989). A major factor regulating flavonoid biosynthesis is the transcriptional activation of phenylpropanoid biosynthetic genes. Chalcone synthase (CHS) catalyzes the first step in the flavonoid-specific branch of the phenylpropanoid pathways. The increase in CHS activity in response to UV light irradiation (Chappel and Hahlbrock, 1984) and the presence of a fungal elicitor (Schmelzer et al., 1984) have been correlated with increased steady-state levels of CHS mRNA.

Recently, we reported the cloning and characterization of CHS genes from soybean (Wingender et al., 1989). Transcriptional regulation was investigated and indicated that soybean *chs* gene expression is induced upon wounding, elicitor treatment, and UV light irradiation. When specifically probing seedling RNA for three different *chs* transcripts, only one gene (*chs*1) was found to be transcribed after both UV light irradiation and elicitor treatment. To define the *cis*-acting elements of the *chs*1 promoter involved in plant defense, we established a regulated transient expression system in soybean protoplasts. We present evidence that the *chs* genes in freshly isolated soybean protoplasts are not immediately inducible by elicitor (Mieth et al., 1985) but regain responsiveness under certain culture conditions. This system enabled us to test chimeric *chs*1-*npt*II/*gus* gene fusions. By functional analysis of 5′ deletions, the sequences of the *chs*1 promoter sufficient to confer regulation by fungal elicitor could be delimited down to 40 bp.

Because parsley protoplasts retain their differential responsiveness to UV light and to fungal elicitor (Dangl et al., 1987), we also tested chimeric deletion mutants of the *chs*1 promoter in the parsley system. As in the case of snapdragon *chs* (Lipphardt et al., 1988), the soybean sequences were correctly recognized in this heterologous system. By in vivo footprinting of the endogenous parsley *chs* promoter (Schulze-Lefert et al., 1989a), *cis*-acting elements involved in light regulation have been defined. A potentially similar function of the homologous soybean *chs* sequences on the basis of the *cis* analysis presented here is discussed.

Because parsley cells accumulate furanocoumarins (but not flavonoids) upon pathogenic attack, the endogenous *chs* is not responsive to elicitor treatment. It was, therefore, of special interest to test whether soybean *chs* sequences would confer elicitor inducibility in parsley protoplasts. We present evidence that the same sequences direct defense regulation in soybean as well as in the heterologous system. This result suggests that parts of the signaling pathway at the level of transcriptional activation of plant defense genes are conserved.

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RESULTS

Elicitor-Inducible Expression of CHS in Soybean Protoplasts

Mieth et al. (1985) showed that soybean protoplasts react in a manner similar to that of intact cells that have been treated with elicitor. Moreover, the protoplasts are unresponsive to further elicitor treatment for up to 70 hr after isolation. A prerequisite for establishing a transient expression system in soybean protoplasts was to find conditions under which they would retain or regain their responsiveness to elicitor treatment. Therefore, we studied the inducibility of CHS by elicitor treatment in protoplasts by varying the following parameters: cell line, cell culture conditions, age of cells, and protoplasting procedure.

We found the most important variables to be harvesting the cells at the mid-logarithmic stage of growth, culture of the cell suspension in Murashige and Skoog (1962) medium, and incubation of the cells for as short a time as possible in the enzyme solution (data not shown). For instance, the age of soybean cell cultures affects their susceptibility to elicitor treatment (Apostol et al., 1989). A similar phenomenon in susceptibility was observed with a second elicitor treatment. Five-day-old to 7-day-old cultures reacted only weakly to elicitor and never regained responsiveness, whereas 3-day-old to 4-day-old cultures did. Therefore, protoplasts were isolated from 3-day-old cell cultures for the transient expression experiments. Figure 1 shows CHS expression at the protein as well as at the steady-state mRNA level in protoplasts at different times after isolation. As predicted by the results of Mieth et al. (1985), protoplasting of soybean cell cultures resulted in a transient induction of CHS, which dropped to a basal level after 12 hr. On the following 2 days, no induction of CHS could be detected following elicitor treatment. However, on the third day of incubation, the cells regained responsiveness to elicitor addition. These data suggest that soybean cells need a recovery phase before they can react to a second elicitor treatment. The time needed for restoration of the responsiveness seems to depend on different parameters such as the cell line and culture conditions used. Although this might be due to a variety of factors, we favor the view that there may be a feedback control by the end product glyceollin or any other metabolite of the biosynthetic pathway and that the parameters mentioned above influence their inactivation.

Sequences up to −219 of a Plant Defense Gene Are Sufficient for Elicitor Inducibility

We reported that two soybean *chs* genes (*chs*1 and *chs*3) are tightly linked in a 5′ to 5′ orientation (Wingender et al., 1989). Hence, it is likely that the intergenic region between *chs*1 and *chs*3 might represent a double promoter. Moreover, *chs*1 was shown to be inducible in soybean seedlings upon UV and elicitor treatment (Wingender et al., 1989). Therefore, we were interested in learning to what extent the intergenic region controls *chs*1 expression. Deletion mutants of the *chs*1 5′ region generated by ExoIII treatment (Wingender et al., 1989) were cloned in the plant expression vector pGDW 44 (Wing et al., 1989). This vector was designed for rapid analysis of putative enhancer sequences placed upstream of a heterologous minimal promoter driving a neomycin phosphotransferase II (NPTII) gene.

Protoplasts were isolated and treated with naked DNA as described in Methods. On the third day of culture, elicitor was added to a final concentration of 1 μg/mL, whereas the controls received water. Eight hours later, the cells were harvested and NPTII activity was determined. Representative NPTII assays of duplicate experiments with different protoplast isolations and DNA preparations are shown in Figure 2. The longest construct (44CS1), spanning the intergenic region between *chs* genes 1 and 3, conferred elicitor inducibility. Deleting to −219 (44CS1Δ1) abolished basal transcription but retained elicitor inducibility. This indicates that sequences necessary for elicitor responsiveness reside between the position −219 and −74. Including the sequences up to −322 (44CS1Δ2) resulted in detectable background activity. However, no increased expression was observed upon elicitor induction. This result might indicate the presence of a regulatory

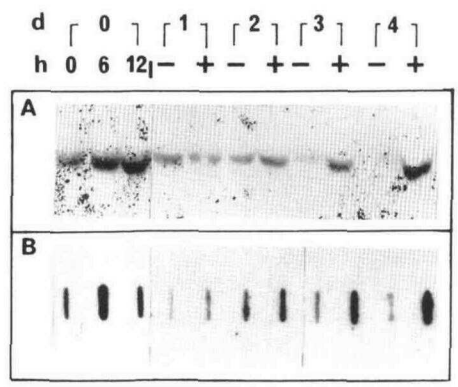

Figure 1. *chs* Expression in Soybean Protoplasts.

Protein and RNA were isolated from soybean protoplasts directly after preparation of protoplasts and at the times indicated on the figure. On the first to fourth day after isolation, the cells were treated with elicitor (+), whereas the controls received water (−).
(A) Protein gel blot analysis (50 μg of total protein/lane) of a typical experiment.
(B) The corresponding RNA slot blot analysis (2.5 μg of total RNA/slot) is shown.
d, day; h, hour.

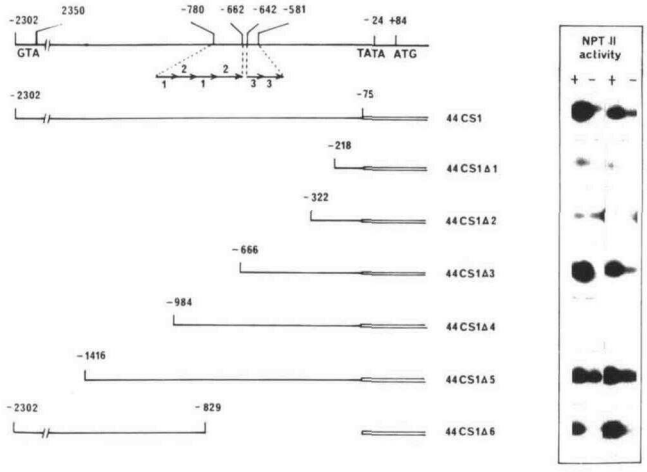

Figure 2. Deletion Analysis of the 5′-Flanking Region of the Chimeric *chs*1/*gene*2/*nptII* Gene.

The diagram presents a schematic drawing of the intergenic region between *chs*1 and *chs*3 together with the deletion series transferred into soybean protoplasts. Translation start points, putative TATA boxes, the position of repeated sequences (named 1, 2, and 3), and deletion endpoints are given as distance in nucleotides from the cap site of the *chs*1 gene (Wingender et al., 1989). Sequences of the *chs*1 promoter replaced by the heterologous minimal promoter are indicated with a double line. The soybean cells were induced 3 days after transformation by the addition of 1 μg/mL elicitor. The NPTII activity exhibited by induced (+) and uninduced (−) cells in two different experiments is shown in the right panel.

element responsible for the background transcription observed in uninduced in vitro cultured cells. Full promoter strength was restored when sequences up to −666 (44CS1Δ3) controlled NPTII expression. This construct contains a 30-bp repeated sequence at position −642 to −581 (Figure 2). A 47-bp repeat located between positions −570 and −670 in the snapdragon *chs* gene (K. Fritze, D. Staiger, I. Czaja, R. Walden, J. Schell, and D. Wing, unpublished results) was shown to have an enhancing function. Comparison of the sequences revealed no striking similarity between these two types of repeats. However, because both types of repeats are located at approximately the same positions in the two *chs* promoters, it could be that the direct 30-bp repeat of the soybean *chs*1 acts as a positive regulatory element of the sequences between −666 and −322. Moreover, the repeat is very AT rich. Such AT-rich simple sequences have unusual stereochemical properties (Wright and Dixon, 1986) and can, therefore, act as upstream enhancer elements (Struhl, 1985). Remarkably, the construct with sequences up to −984 (44CS1Δ4) had no transcriptional activity, although this construct contains all repeats and the elements required for elicitor induction. On the other hand, a high level of background transcriptional activity that is not influenced by elicitor was observed when sequences up to −1416 (44CS1Δ5) controlled NPTII expression.

These results cannot be explained in a simple model. Apparently, the architecture of this promoter is complex, possibly as a consequence of the close head-to-head orientation of two *chs* genes. This complexity is further illustrated by the fact that deletion of sequences between −829 and −75, containing elements necessary for elicitor induction, background activity, and enhancement (44CS1Δ6), is nevertheless fully active. This result suggests that the region between −2302 to −829 can act as an alternative promoter or that the exceptional configuration of *chs*1 and *chs*3 with their divergent transcription leads to regulatory elements capable of influencing transcription in both directions, particularly when deletions are considered. These results probably also reflect the cooperativity between different *cis*-acting elements. This has already been reported for other genes (Stougaard et al., 1987; Kuhlemeier et al., 1988; Schöffl et al., 1989), where differences in expression result from the complex interplay between different quantitative and qualitative elements within their promoters. It is tempting to speculate that the drastic changes in transcriptional activity upon deletion mutation observed for CS1 constructs might be due to the activity of elements that normally function in the expression of *chs*3 coming into play.

Conserved *chs* Promoter Regions Serve as *cis*-Acting Elements in Elicitor Induction

From these results, we concluded that the minimal sequences sufficient for elicitor inducibility are located on the Δ1 construct. To delimit the elicitor responsive sequences more precisely, a new series of deletion mutants was designed. Because the short constructs expressed NPTII only poorly, a more sensitive marker, the β-glucuronidase (GUS) gene, was used as reporter gene. In addition, the minimal promoter was exchanged with the original 5′ region of *chs*1 in a transcriptional fusion to the *gus* gene (for details, see Methods).

Previous sequence comparison of three *chs* genes (Wingender et al., 1989) showed the presence of conserved boxes in the 5′ region of these genes. To test whether these regions are involved in the transcriptional activity of the *chs* promoters, the deletions were introduced in a way that they cut into the middle of these regions. A schematic drawing of the constructs is depicted at the bottom of Figure 3. The smallest construct (CS1Δ1c) covers sequences up to −75 cutting in the middle of box II. CS1Δ1b spans the region up to −134, including box II and half of box I. In transient expression in soybean protoplasts, the smallest construct showed GUS activity slightly above

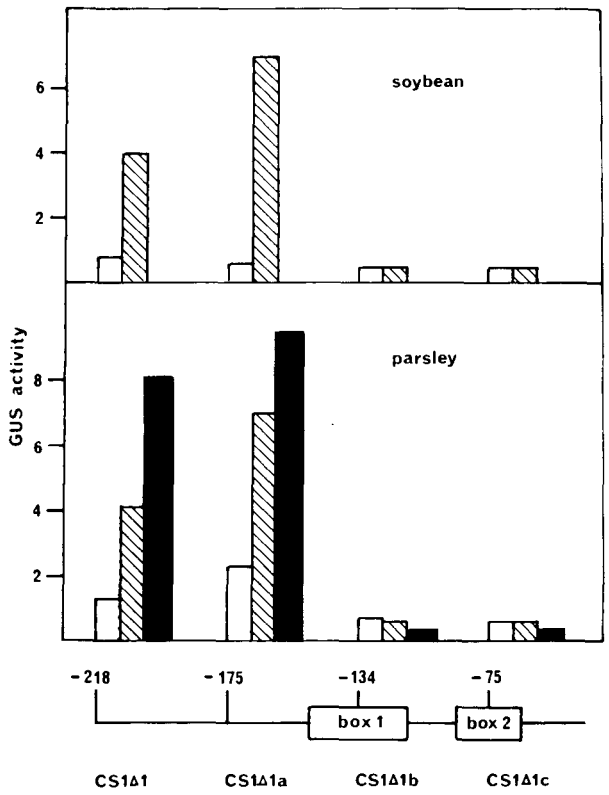

Figure 3. Elicitor-Inducible and UV Light-Inducible GUS Expression Driven by *chs*1/*gus* 5′ Deletion Mutants in Soybean and Parsley Protoplasts.

A schematic drawing of the *chs*1 promoter region is depicted at the bottom, indicating the positions of the deletions and the conserved regions (box 1, box 2) of the *chs* genes (Wingender et al., 1989). The corresponding GUS activity exhibited by transformed uninduced (open bars), elicitor-induced (hatched bars), and UV light-irradiated (black bars) cells is shown in the upper panel. The amount of error in soybean protoplasts was ±0.3 and in parsley protoplasts, ±0.15.

background, which was also true when sequences up to −134 were present. Elicitor inducibility was only observed when sequences up to −175 (CS1Δ1a) controlled GUS expression. Interestingly, the region between −175 and −218 seemed to down-regulate transcription rate. These findings support the proposal that box I is involved in elicitor-induced transcriptional activation.

cis-Acting Elements Involved in Plant Defense Retain Their Function in a Novel Host

Parsley protoplasts retain their responsiveness to fungal elicitor and are not elicited by the protoplasting procedure (Dangl et al., 1987). This system, therefore, provides a useful tool for the analysis of the assumed elicitor-responsive *cis*-elements of soybean. The deletion mutants CS1Δ1 to CS1Δ1c were transferred to parsley protoplasts, and GUS activity was measured in elicited and control cells 6 hr after DNA transfer. As shown in Figure 3, in parsley protoplasts the same results were obtained as in soybean protoplasts, indicating that the soybean *cis*-acting elements are correctly recognized in the heterologous parsley system. These data suggest that common features in the signaling pathway at the level of transcriptional activation of plant defense genes are conserved.

UV Light Inducibility of Chimeric CHS Constructs in Parsley Protoplasts Depends on Two *cis*-Acting Elements

Parsley protoplasts are UV light responsive (Dangl et al., 1987). Therefore, we tested the UV light inducibility of the chimeric *chs*1/*gus* gene constructs mentioned above in parsley protoplasts because soybean cell cultures or protoplasts are not responsive to UV irradiation (data not shown). As shown in Figure 3, the constructs CS1Δ1 and CS1Δ1a were inducible upon continuous UV light irradiation, whereas CS1Δ1b and CS1Δ1c were not. This demonstrates that the same sequences are sufficient and necessary for UV and for elicitor induction, suggesting that UV-responsive and elicitor-responsive regulatory elements are physically linked.

Nevertheless, there is a marked difference between the two induction systems. Figure 1 shows that the use of a minimal promoter of the T-DNA indole-3-acetamide hydrolase gene did not abolish elicitor inducibility in soybean

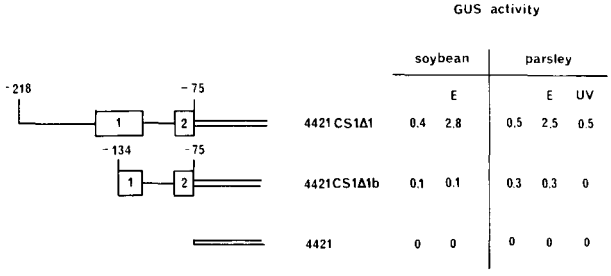

Figure 4. Elicitor-Inducible and UV Light-Inducible GUS Expression of Chimeric *chs*1 /*gene2*/*gus* Gene Fusions in Soybean and Parsley Protoplasts.

*chs*1 promoter sequences corresponding to CS1Δ1 and CS1Δ1b (see Figure 3) were moved to the vector pGDW 4421, resulting in the replacement of sequences downstream of −75 by the *gene2* minimal promoter (indicated by a double line). Soybean and parsley protoplasts were transformed with these constructs and the vector alone. GUS activity was determined after UV or elicitor treatment.

protoplasts. To test whether this is also true for UV light inducibility in parsley protoplasts, new constructs corresponding to CS1Δ1 and CS1Δ1b were constructed in the vector pGDW4421. As depicted in Figure 4, the construct 4421CS1Δ1 conferred elicitor inducibility in soybean and in parsley protoplasts, whereas deleting down to −134 (4421CS1Δ1b) resulted in loss of inducibility. Surprisingly, no UV light inducibility of 4421CS1Δ1 was observed in the parsley system. This indicates that in addition to the region between −175 and −134, sequences downstream of −75 are involved in UV light but not in elicitor responsiveness in parsley protoplasts.

DISCUSSION

We report here that soybean protoplasts regain elicitor inducibility of *chs* genes after a variable recovery phase depending on culture conditions. This finding raises the question of whether the signal transduction pathway is distorted or whether the genes after transient induction are repressed before the recovery. Soybean microsomal fractions as well as protoplasts are able to specifically bind fungal β-glucans (Cosio et al., 1988). Therefore, it is not likely that freshly isolated protoplasts lack elicitor responsiveness because the presumed elicitor receptor is missing. In transient expression experiments with noninducible protoplasts (1 day to 2 days after isolation), we always observed less expression of the chimeric *chs* gene in elicitor-treated cells than in the controls (data not shown). This indicates that the protoplasts are, in fact, responsive to the elicitor and that the genes involved in phytoalexin production are down-regulated during the recovery phase. A regulated transient expression system in electroporated soybean protoplasts was described previously (Dron et al., 1988). In this system, protoplasts were responsive to glutathione 24 hr after protoplast isolation, indicating that the length of the recovery period might vary between different cell cultures.

The establishment of a regulated transient expression system in soybean protoplasts enabled us to perform a 5′ deletion analysis of the *chs*1 promoter. The differences in transcriptional activity and inducibility between the deletion mutants can most probably be attributed to a complex arrangement of regulatory elements within the promoter. This is likely to result from the 5′ to 5′ orientation of *chs* genes 1 and 3, suggesting either interaction of regulatory elements from the two promoters or the presence of a double promoter. Our results indicate that the sequences −175 to −134 are necessary for elicitor inducibility. Their possible function as *cis*-acting element is further supported by the fact that they cover a region of high homology between different soybean *chs* genes (Wingender et al., 1989).

By functional analysis of truncated *chs* promoter fusions in parsley protoplasts, a minimal light-responsive promoter could be defined. The sequences necessary for UV light activation of the parsley *chs* gene were defined by in vivo DNA footprints and *cis* analysis (Schulze-Lefert et al., 1989a, 1989b). The parsley box II CCACGTGGCC covers a sequence highly conserved among other light-regulated plant genes (Giuliano et al., 1988; Staiger et al., 1989). Even in the *Arabidopsis Adh* gene, which is not regulated by light, a related box is present (McKendree et al., 1990). Both snapdragon and parsley *chs* promoters have the core CACGTG motif located within the UV light-responsive region (Kaulen et al., 1986; Lipphardt et al., 1988; Schulze-Lefert et al., 1989a, 1989b). Mutation of the parsley box II results in a loss of UV light-responsive expression (Schulze-Lefert et al., 1989a). A similar sequence, CTACGTAGCC, can be found in the soybean *chs* promoter at position −140. Deleting half of this sequence (construct CS1Δ1b) abolishes light responsiveness. CS1Δ1a contains this sequence in its entirety and can be induced by UV light, indicating that this region might be involved in UV responsiveness. The *trans*-acting factor CG-1 (Staiger et al., 1989), recognizing a related sequence from the snapdragon *chs* promoter, the box II of parsley *chs*, and the G-box of genes encoding the small subunit of ribulose-1,5-bisphosphate carobxylase (Giuliano et al., 1988), does not bind to the soybean sequence (Staiger et al., 1989). Until now, it has been unclear whether these related sequences act as qualitative or quantitative elements and whether they bind the same *trans*-acting factor. It would be of considerable interest, therefore, to test whether the parsley box II binding factor can bind to the soybean sequence.

Because replacement of soybean sequences downstream of −75 by a heterologous minimal promoter abolished light regulation, TATA-proximal sequences must be involved in UV responsiveness. This suggests that, as in the case of the parsley *chs* promoter (Schulze-Lefert et al., 1989a), the soybean *chs* promoter contains two *cis*-acting elements involved in light regulation. A sequence related to the necessary parsley box I (AACCTAACCT) can be found at position −42—TACCTACCAA—of the soybean promoter, suggesting, in agreement with the *cis* analysis, a possible involvement of this region in light regulation.

Our results indicate that the *cis*-acting elements involved in plant defense are functionally conserved between species because they seem to be recognized by the appropriate *trans*-acting factors in a heterologous host. In this context, it should be noted that the endogenous parsley *chs* gene is not involved in the synthesis of phytoalexins and is not elicitor responsive. In parsley, phenylalanine ammonia lyase (PAL) genes readily respond to different types of stress, such as UV light irradiation, elicitor treatment, or wounding. In vivo footprinting of the parsley *pal*1 promoter demonstrated elements thought to be involved

in the responses to UV light and elicitor (Lois et al., 1989). The only striking sequence similarity of the soybean promoter to these footprinted regions was in a region homologous to the parsley box I sequence mentioned above. Deletion of this element (4421 constructs) from the soybean promoter, however, did not abolish elicitor responsiveness in either protoplast system, suggesting that, in contrast to the UV light response, this region might not be involved in elicitor regulation. So far the only similarity between the parsley *pal*1 and the soybean *chs*1 promoters seems to be that UV-responsive and elicitor-responsive elements might be relatively close.

Data from 5′ promoter deletions of the French bean *chs*15 promoter suggest the presence of an elicitor-responsive sequence between the TATA box and position −130 (Dron et al., 1988). The significance of these data is enhanced by the finding of two elicitor-inducible DNase I hypersensitive sites in this region (Lawton et al., 1990). However, no significant sequence homologies to the equivalent elements in the soybean *chs* promoter could be found.

Both parsley and soybean are responsive to crude elicitor preparations from the *Phytophthora megasperma* f. sp *glycinea* cell wall; however, the elicitor molecules are different. In the case of soybean, the active molecule is a β-glucan, whereas parsley recognizes a protein (Parker et al., 1988). This suggests that regardless of the signal molecule, parts of the signaling pathway, i.e., factors involved in the transcriptional regulation of plant defense genes, are conserved. The lack of absolute sequence homology between the *cis*-elements involved in plant defense described so far might indicate that mere sequence comparison is not sufficient for the identification of the relevant regions. Future work will concentrate, therefore, on the identification of protein binding domains involved in plant defense of the soybean *chs*1 promoter.

METHODS

Recombinant DNA Techniques

Cloning steps were performed according to Maniatis et al. (1982). To generate different deletion mutants of the *chs*1 promoter, the 3-kb KpnI fragment of pGm11,4a (Wingender et al., 1989) covering the intergenic region between *chs*1 and *chs*3 was subcloned into M13mp18 (Norrander et al., 1983). Using unidirectional ExoIII digestion (Henikoff, 1984), regions 5′ to the *chs*1 gene were deleted to varying extents and the resulting deletion mutants sequenced. Several mutants were chosen for further analysis, cloned as SphI fragments into the vector DOVE, and subsequently transferred to the plant expression vectors pGDW 44 or pGDW 4421 (Wing et al., 1989). pGDW 44 provided a neomycin phosphotransferase (NPTII) and, in the case of pGDW 4421, a β-glucuronidase (GUS) gene fused to a minimal promoter of the T-DNA indole-3-acetamide hydrolase gene. These vectors allowed the analysis of positive regulatory sequences fused to the minimal promoter. According to the vector used, the constructs were named 44CS1 or 4421 CS1. Constructs carrying the original TATA box and leader sequence of *chs*1 were cloned in pUC19 (Yanisch-Perron et al., 1985). For this purpose, the *gus* gene from pBI101.1 (Jefferson, 1987) was cloned as a HindIII-EcoRI fragment into the appropriate restriction sites of pUC19. Subsequently, the 5′ region of *chs*1 was cloned as a SphI-AluI fragment covering positions −75 to +79 (ATG at position +84) into the SphI and SmaI sites in front of the *gus* gene. The deletion derivatives described above were then fused as EcoRI-SphI fragments to the chimeric *chs*1/*gus* gene.

Plant Material and Transient Expression

The soybean suspension culture was derived from callus material of *Glycine max* cv Harsoy, which was kindly provided by Prof. H. Barz, University of Münster. The suspension was grown in the dark on a rotary shaker at 26°C in 40 mL of Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1 mg of 2,4-dichlorophenoxyacetic acid per liter. For protoplast isolation, 3-day-old to 4-day-old cultures were harvested and the cells suspended in 10 mL of a solution (pH 5.5) containing 0.6 M mannitol, 1% cellulase "Onozuka" RS, 0.5% macerozyme R-10 (both enzymes from Yakult Honsha, Nishinoma, Japan), and 0.05% pectolyase (Sigma). The suspension was incubated for 2 hr with gentle shaking, and protoplasts were isolated by passing the cells successively through 300-μm, 100-μm, and 50-μm sieves. Protoplasts were washed twice with sterile seawater, centrifuged, and then resuspended at a final concentration of 2×10^6 cells per 0.24 mL in B5 medium (Gamborg et al., 1968) supplemented with mannitol to give a final osmolarity of 700 mOsmol. The protoplasts were transformed via the $Ca(NO_3)_2$/PEG method (Krens et al., 1982). Drops (0.24 mL) of cells were mixed with 40 μL of DNA solution (20 μg of salmon sperm DNA, 20 μg of plasmid), and 200 μL of PEG solution (25% PEG 6000, 0.45 M mannitol, 0.1 M $Ca(NO_3)_2$, pH 9) were added. After 20-min incubation, 5 mL of 0.275 M $Ca(NO_3)_2$ (pH 6) were added and the protoplasts pelleted 20 min later by centrifugation. The pellet was resuspended in 5 mL of B5 medium supplemented with 0.6 M mannitol, 0.1 mg of 2,4-dichlorophenoxyacetic acid, 0.1 mg of 1-naphthaleneacetic acid, and 0.1 mg of 6-benzyladenine per liter.

Parsley suspension cultures were kindly provided by Dr. D. Scheel, MPI Köln, and grown as described (Hahlbrock, 1975). Isolation of parsley protoplasts was performed as described previously (Dangl et al., 1987). Protoplasts were transformed by the method developed by Negrutiu et al. (1987). The isolated protoplasts were adjusted to a concentration of 1.6×10^6 cells/mL in MaMg (0.4 M mannitol, 15 mM $MgCl_2$, 0.1% morpholinoethanesulfonic acid, pH 5.6). Drops (0.3 mL) were mixed with 40 μL of DNA (20 μg of salmon sperm DNA, 20 μg of plasmid) and 0.3 mL of PEG CMS [40% PEG 6000, 0.4 M mannitol, 0.1 M $Ca(NO_3)_2$, pH 7]. After 25-min incubation, 5 mL of 0.24 M $CaCl_2$ were added and the protoplasts were pelleted by centrifugation. The pellet was resuspended in 2 mL of B5 medium supplemented with 0.4 M sucrose and 1 mg of 2,4-dichlorophenoxyacetic acid per liter.

Elicitor Treatment and UV Light Irradiation

β-Glucan elicitor was added to soybean cells to a concentration of 1 μg/mL, and the cells were harvested after 8 hr of incubation

by centrifugation. The β-glucan elicitor was kindly provided by Prof. H. Reisener, University of Aachen. Parsley protoplasts were treated with crude elicitor derived from the cell wall of *Phytophthora megasperma* f sp *glycinea* (50 μg/mL) (Ayers et al., 1976) directly after transformation and incubated for 6 hr. UV light irradiation was performed as described previously (Dangl et al., 1987). The samples were frozen in liquid N_2 and stored at −70°C.

Enzyme Assays

NPTII activity was determined as described (Reiss et al., 1984), and GUS activity followed the protocol of Jefferson (1987). GUS activity throughout this work is expressed in micromoles of methylumbelliferone per milligram of protein per minute.

Electrophoresis and Immunoblotting

$NaDodSO_4$/PAGE was performed in 12% polyacrylamide gels (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose (Towbin et al., 1979), and the membranes were incubated with low-fat milk powder in phosphate buffered saline before incubation with antibody directed against soybean CHS, which was kindly provided by Prof. H. Grisebach, University of Freiburg. The membranes were washed and subsequently incubated with phosphatase-conjugated goat anti-rabbit IgG antisera (Bio-Rad).

RNA Preparation

RNA was prepared and slot blot analysis was performed as described previously (Wingender et al. 1989).

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