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Glutathione and fungal elicitor regulation of a plant defense gene promoter in electroporated protoplasts

(chalcone synthase/elicitor induction/transcription regulation)

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ABSTRACT **To investigate the mechanisms underlying activation of plant defenses against microbial attack we have studied elicitor regulation of a chimeric gene comprising the 5′ flanking region of a defense gene encoding the phytoalexin biosynthetic enzyme chalcone synthase fused to a bacterial chloramphenicol acetyltransferase gene. Glutathione or fungal elicitor caused a rapid, marked but transient expression of the chimeric gene electroporated into soybean protoplasts. The response closely resembled that of endogenous chalcone synthase genes in suspension cultured cells. Functional analysis of 5′ deletions suggests that promoter activity is determined by an elicitor-regulated activator located between the "TATA box" and nucleotide position −173 and an upstream silencer between −173 and −326. These cis-acting elements function in the transduction of the elicitation signal to initiate elaboration of an inducible defense response.**

Plants respond to microbial attack by synthesis of antibiotics, stimulation of lytic enzymes, and reinforcement of cell walls (1–4). These defenses can also be induced by glycan and glycoprotein elicitors from fungal cell walls and culture fluids or metabolites such as arachidonic acid and glutathione (1–5). Elicitors, wounding, or infection rapidly stimulates the transcription of genes involved in the erection of these defenses (5–10). To investigate this early event in the activation of resistance mechanisms we have studied the expression in electroporated soybean protoplasts of a chimeric gene comprising the 5′ flanking region of a defense gene encoding chalcone synthase (CHS) fused to a bacterial chloramphenicol acetyltransferase (CAT) gene and the 3′ flanking region of the nopaline synthase (NOS) gene.

CHS catalyzes the condensation of 4-coumaroyl-CoA with three acetate units from malonyl-CoA to give naringenin chalcone. This is the first step in a branch of phenylpropanoid metabolism specific for the synthesis of isoflavonoid phytoalexin antibiotics in legumes and flavonoid pigments that are ubiquitous in higher plants (2, 11). Elicitor stimulates CHS transcription in bean cells within 5 min, leading to a transient accumulation of CHS mRNA with maximum levels after 3–4 hr, correlated with the onset of phytoalexin synthesis (7, 9, 12).

We show here that glutathione or a fungal elicitor preparation of high molecular weight material heat-released from mycelial cell walls of the bean pathogen *Colletotrichum lindemuthianum* (fungal elicitor) causes a rapid, marked but transient expression of the chimeric CHS-CAT-NOS gene electroporated into soybean protoplasts. The response of the CHS-CAT-NOS gene closely resembles that of endogenous CHS genes in elicitor-treated cell suspension cultures. The data show that the 429-base-pair (bp) nucleotide sequence immediately upstream of the CHS coding region is sufficient to confer regulation by glutathione or fungal elicitor. Functional analysis of 5′ deletions suggests that transduction of the elicitation signal to initiate elaboration of inducible defenses involves an elicitor-regulated activator located between the "TATA box" and −173 and an upstream silencer between −173 and −326.

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MATERIALS AND METHODS

Plasmid Constructions. pDO400 is identical to the previously described cauliflower mosaic virus (CaMV) 35S promoter construct pDO432 (13) except that an 883-bp *Bam*HI fragment containing the *Escherichia coli* CAT gene (14) replaces the luciferase reporter gene of pDO432. pCHS15 consists of a 2.1-kilobase (kb) *Hin*dIII *Phaseolus vulgaris* genomic fragment containing the CHS 15 gene and flanking sequences subcloned into the riboprobe vector pSP64 (15). In pCHC1, a 429-bp *Hin*fI fragment comprising 5′ untranslated sequences of CHS 15 replaces the 35S transcript promoter of pDO400.

pCHC1 was constructed by replacing the *Hin*dIII/*Xba* I CaMV 35S promoter fragment of pDO400 with the *Hin*dIII/*Xba* I polylinker fragment of pUC19 to create pCN100. pCN100 was digested with *Sal* I, filled in with Klenow DNA polymerase and dNTPs, and used for blunt-end ligation of the 429-bp *Hin*fI fragment of pCHS15, whose ends were similarly rendered blunt by Klenow fill-in. The construct was sequenced by dideoxy chain-termination (16) of denatured double-stranded plasmid with an M13 reverse primer (17). Deletion mutants were constructed by digesting pCHC1 with *Hin*dIII followed by exonuclease III and mung bean nuclease treatment (18). After *Xba* I digestion, deleted promoter fragments were purified on low-melting agarose and ligated into *Pst* I (T4 polymerase filled-in)/*Xba* I-cut pCN100. Precise endpoints were determined by sequencing as described above. pHCN1 was constructed by cloning a 235-bp *Eco*RI/*Pvu* II fragment from the promoter region of a murine histone H4 gene (19) into *Eco*RI/*Sma* I-cut pIBI24 (a pUC-derived phagemid vector). This construct was further cleaved with *Eco*RI/*Xba* I and subcloned into *Hin*dIII/*Xba* I-digested pCN100 along with the entire *Eco*RI/*Hin*dIII polylinker from pIBI24.

Protoplast Isolation. The origin and maintenance of bean (*P. vulgaris* L.), soybean (*Glycine max* L.), and tobacco (*Nicotiana tabacum* L.) cell suspension cultures were as

Abbreviations: CAT, chloramphenicol acetyltransferase; CHS, chalcone synthase; NOS, nopaline synthase; PAL, phenylalanine ammonia-lyase; CaMV, cauliflower mosaic virus.

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described except that cells were collected by sieving (250-μm mesh) and transferred to fresh maintenance medium at 7-day intervals (20, 21). For protoplast isolation, cells (7 g fresh weight) were collected 4 days after subculture and incubated by shaking (90 rpm) in 100 ml of protoplast isolation medium for 4 hr at 27°C in darkness (22). Protoplasts were separated from the cellular debris by sieving and by centrifugation at 70 × g for 5 min at room temperature. Viability was determined by staining with Evans blue and protoplasts were adjusted to 5×10^6 per ml. Protoplasts were washed twice in electroporation medium (22) prior to manipulation.

Electroporation and Transient Assay. Electroporation was performed as described (22) 3 hr after isolation of protoplasts using an optimal pulse of 250 V for 10 msec. Unless otherwise noted, 30 μg of test construct DNA was electroporated together with 50 μg of calf thymus DNA as carrier. Protoplasts were maintained without agitation in 6 ml of maintenance medium containing 0.3 M mannitol at 27°C in the dark. In the experiment depicted in Fig. 2A, protoplasts were collected for analysis 8 hr after electroporation. In all other experiments, protoplasts were incubated for 21 hr after electroporation prior to addition of a fungal elicitor preparation heat released from mycelial cell walls of *C. lindemuthianum* (fungal elicitor, ref. 20) or glutathione (5) in maintenance medium containing 0.3 M mannitol. Final concentrations of fungal elicitor and glutathione were 60 μg of glucose equivalents/ml and 1 mM, respectively. Equal volumes of maintenance medium containing 0.3 M mannitol were added to control protoplasts. Protoplasts were collected by centrifugation, and extracts were assayed for CAT activity by radiometric measurement of the conversion of the substrate [^{14}C]chloramphenicol as described (22). Reaction products were separated by thin-layer chromatography, visualized by autoradiography, and quantitated by scintillation spectroscopy. Protein was assayed by the Bradford procedure (23). Typical CAT assays involved incubation of samples containing 5 μg of protein for 3 hr at 37°C leading to the conversion of 1000–5000 cpm of the substrate into acetylated products.

RNA Analysis. Protoplasts (3×10^6) were resuspended in 100 μl of 0.1 M Tris·HCl (pH 9.0) containing 0.01% sodium dodecyl sulfate (SDS). After extraction with phenol/chloroform, the supernatant was precipitated with 2 vol of 95% ethanol in the presence of 0.3 M sodium acetate. RNA was further processed and analyzed by transfer blot hybridization as described (20). The hybridization probe was a 0.8-kb *Bam*HI fragment comprising *E. coli* CAT gene sequences (14) labeled by nick-translation.

RESULTS

To analyze CHS promoter function, the expression of a chimeric gene comprising the 5′ flanking region of the CHS 15 gene fused with the coding sequences of CAT and the 3′ flanking sequences of NOS (Fig. 1) was examined following electroporation into protoplasts derived from suspension cultured cells. CHS 15 is one of six CHS genes in the bean genome and encodes a major elicitor-induced CHS transcript (15). The chimeric CHS-CAT-NOS gene construct pCHC1 contains 429 bp of the 5′ untranslated nucleotide sequences of CHS 15, comprising 326 bp upstream of the transcription start site and 103 bp of the transcribed leader sequence (Fig. 1).

As recently reported for parsley (24), bean and soybean protoplasts respond to elicitor in a manner similar to the suspension cultured cells from which they were derived with respect to the accumulation of transcripts encoded by endogenous defense genes and the appearance of phenylpropanoid products (data not shown). However, electroporated bean protoplasts showed only low viability and weak expression of the CHS-CAT-NOS gene compared to tobacco and soybean protoplasts (Fig. 2A). The latter, being closely related to bean, were the major focus for elicitor regulation studies.

To minimize induction by endogenous elicitors and other stress factors released during protoplast preparation (24, 25), freshly isolated protoplasts were incubated for 3 hr prior to electroporation and for a further 21 hr before elicitation. Following addition of glutathione, a marked increase in the level of CAT activity was observed within 3 hr, whereas in untreated controls there was no significant change in CAT activity in this period (Fig. 2B). RNA transfer blot hybridization with nick-translated CAT gene sequences as a probe demonstrated that glutathione stimulation of CAT activity reflected induction of CAT transcripts (Fig. 3). Hence induction of CAT activity could be correlated with stimulation of the transcription of the CHS-CAT-NOS gene. In contrast, glutathione did not modulate CAT activity in protoplasts electroporated with a chimeric gene comprising the promoter of the murine histone H4 gene fused with the CAT-NOS reporter cassette (Fig. 2B).

The response of the CHS-CAT-NOS gene was highly reproducible when different samples of a protoplast preparation were independently electroporated and induced (Fig. 2B). Optimal elicitor regulation was observed with 30–50 μg of the chimeric gene (Fig. 4). Electroporation of larger amounts of the construct resulted in high levels of expression in control protoplasts and correspondingly weak regulation by glutathione. Fungal elicitor also induced expression of the chimeric gene (Fig. 2C), although, as with endogenous CHS genes in suspension cultured cells, the response was somewhat weaker than with glutathione (5).

The CHS-CAT-NOS gene was transiently expressed with maximum levels 3 hr after addition of glutathione followed by a decay to relatively low levels after 6 hr (Fig. 2D). No induction of CAT activity was observed over this period in the absence of glutathione. The chimeric gene was also regulated by glutathione when electroporated into protoplasts derived from tobacco cells, although the response was slower, with maximum CAT activity after 6 hr (Fig. 2D). These induction kinetics closely resembled those for expression of endogenous defense genes in the respective suspension cultured cells from which the protoplasts were derived (refs. 12 and 26; M. G. Hahn and C.J.L., unpublished observations).

These data showed that sequences to −326 of CHS 15 were sufficient to confer regulation by glutathione or fungal elicitor. Deletion from −326 to −173 increased the basal level of CAT activity in soybean protoplasts prior to addition of an external stimulus and moreover caused a striking increase in the response to glutathione (Fig. 5). In contrast, further deletion to −130 reduced basal and induced expression back to about the same respective levels observed with the entire promoter. Deletion to −72 reduced expression in glutathione-treated protoplasts to the basal level observed in unstimulated, control protoplasts. This deletion, which abolishes glutathione regulation, provides an additional internal control for the specificity of induction in the transient assay, since in this construct CHS promoter sequences are replaced by vector sequences adjacent to a functional TATA box. Deletion to −19, which removes the TATA box (−29 to −21), completely abolished expression of the chimeric gene in control and glutathione-treated protoplasts.

These 5′ deletions had similar relative effects on induction by the fungal elicitor preparation (data not shown). Thus, deletion to −173 likewise increased the response to fungal elicitor, although this enhanced induction was somewhat weaker than that obtained with the same construct in response to glutathione. As with glutathione, further deletion to −136 and −72 progressively reduced the response to fungal elicitor.

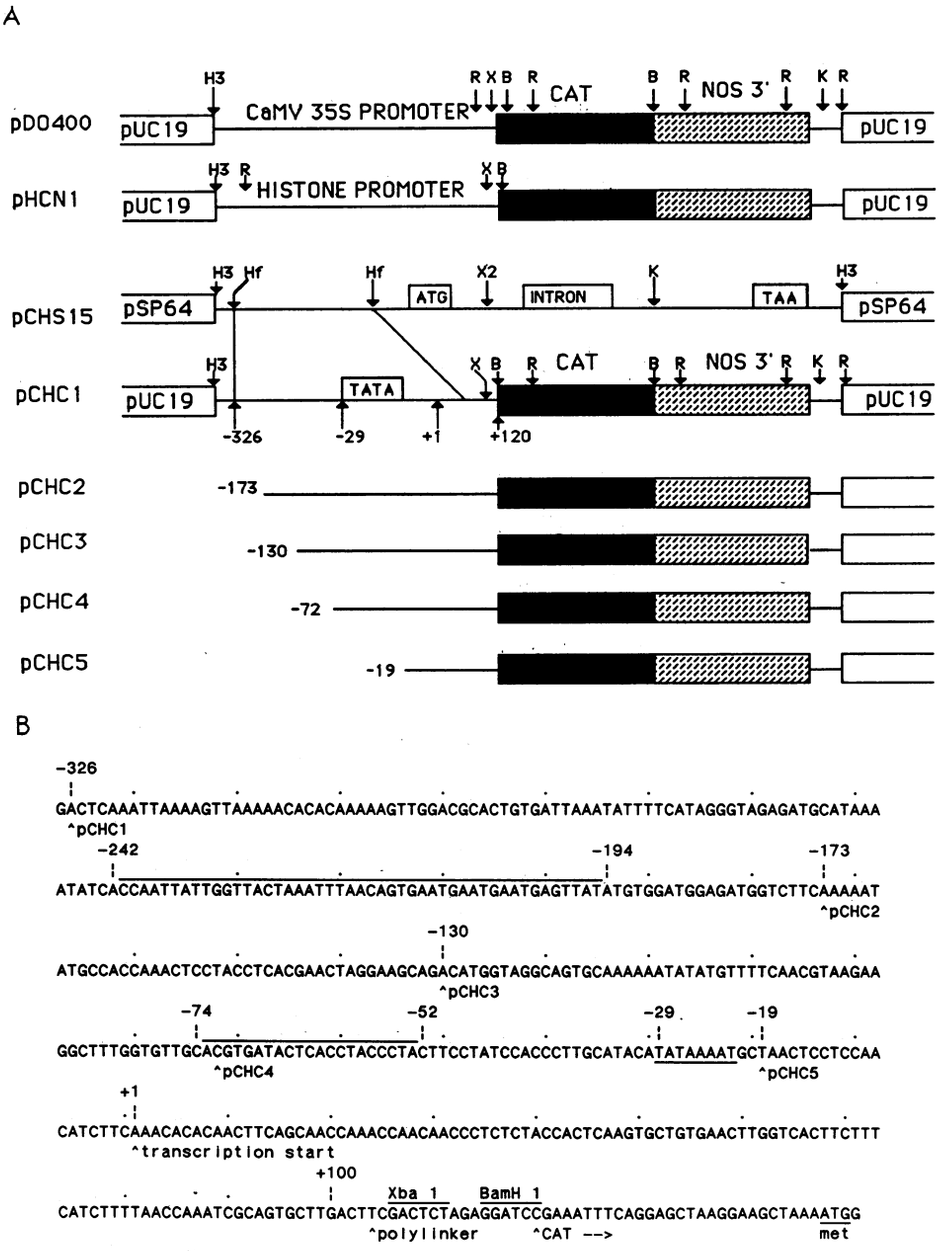

FIG. 1. (*A*) Structure of the CHS-CAT-NOS construct and deletion mutants. (*B*) Nucleotide sequence of the CHS 15 promoter and CAT fusion junction. Restriction sites indicated are B, *Bam*HI; H3, *Hin*dIII; Hf, *Hin*fI; K, *Kpn* I; R, *Eco*RI; X, *Xba* I; X2, *Xho* II. Deletion mutants are marked by arrows. The TATA box is underlined. Sequences conserved in the promoter of an elicitor-induced bean phenylalanine ammonia-lyase (PAL) gene are overscored.

DISCUSSION

The present data show that the CHS promoter is appropriately regulated by glutathione and fungal elicitor in electroporated protoplasts. As in other transient expression systems, it is probable that the CHS-CAT-NOS gene is not inserted into chromosomal DNA and that our experiments monitor the expression of a plasmid-borne gene. However, the response of the chimeric CHS-CAT-NOS gene electroporated into protoplasts closely resembles that of endogenous chromosomal CHS genes in elicitor-treated cell suspension cultures with respect to the kinetics of induction and the relative potency of glutathione and fungal elicitor as inducers. Hence the protoplast system described here provides a convenient functional assay for analysis of cis-acting nucleotide sequences involved in elicitor regulation of defense genes.

The initial studies with a set of nested 5′ deletions suggest that there is an elicitor-regulated activator element downstream from −173. Since 5′ deletions to −130 and to −72 affect elicitor regulation by inhibition of induction rather than by elevation of basal expression, the activator appears to be a positive cis-acting element. This functional analysis is consistent with the pattern of sites hypersensitive to DNase I digestion in CHS genes (M.A.L. and C.J.L., unpublished). Three such sites, which denote local opening of chromatin structure associated with binding of regulatory proteins, are found in the proximal region of the promoter in nuclei from elicitor-treated but not control cells. In contrast, sites in the upstream region show pronounced DNase I hypersensitivity in nuclei from uninduced as well as elicited cells.

Although sequences between the TATA box and −130 are both necessary and sufficient for regulation by glutathione or fungal elicitor, upstream sequences appear to modulate

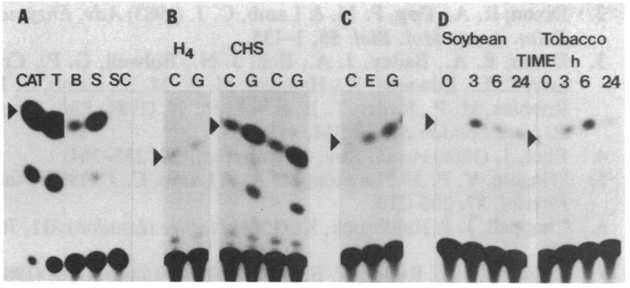

FIG. 2. Expression of the chimeric CHS-CAT-NOS gene electroporated into protoplasts derived from suspension cultured cells. (*A*) Comparison of expression in bean, soybean, and tobacco protoplasts. (*B*) Effect of glutathione on the expression of CHS-CAT-NOS and H4-CAT-NOS chimeric genes in soybean protoplasts. (*C*) Comparison of the induction by fungal cell wall elicitor and glutathione. (*D*) Time course for glutathione-induced expression in soybean and tobacco protoplasts. CAT, authentic bacterial CAT enzyme; T, tobacco; B, bean; S, soybean; SC, soybean protoplasts without electroporated genes; G, protoplasts 3 hr after treatment with glutathione; E, protoplasts 3 hr after treatment with fungal elicitor; C, equivalent, untreated control protoplasts. Closed arrowheads denote the major CAT product, 3-acetylchloramphenicol.

expression, and maximum induction is obtained when sequences to −173 are present. This may reflect the existence of multiple cis-acting sequences that interact with the same trans-acting factor(s) or an independent regulatory element between −173 and −130 that is distinct from the downstream element. Alternatively, deletion of the nucleotide sequences between −173 and −130 may have an impact on gene expression not by abolition of the binding of trans-acting factors to cis-acting elements located in this region but through indirect effects on chromatin structure that modulate binding of transcription factors to the activator element downstream of −130.

Similar indirect rearrangements of chromatin structure might likewise account for the enhanced expression observed by deletion from −326 to −173. However, it is likely that this enhanced expression reflects the removal of a discrete cis-acting silencer element located between −326 and −173. Thus we have recently detected specific binding of a nuclear factor to this region, and moreover, coelectroporation of the putative silencer element in trans with the complete CHS-CAT-NOS gene (pCHC1) leads to a marked stimulation of expression, presumably by competition for binding of the corresponding trans-acting repressor (27). Functional analysis of the nested 5′ deletions does not indicate whether the putative silencer is elicitor regulated, although synergistic

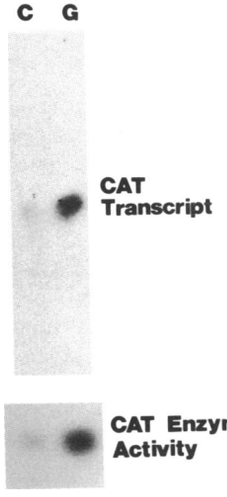

FIG. 3. Correlation between the accumulation of CAT transcripts and CAT activity in electroporated protoplasts containing the CHS-CAT-NOS gene. (*Upper*) Transfer blot of equal amounts of total cellular RNA from control protoplasts (C) or 3 hr after treatment with glutathione (G) hybridized with CAT sequences. (*Lower*) CAT activity from extracts of equivalent protoplasts.

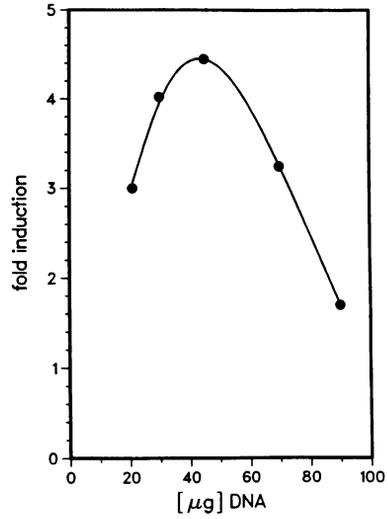

FIG. 4. Glutathione induction of CHS-CAT-NOS relative to basal levels of expression as a function of the amount of the chimeric construct electroporated.

interaction between positive and negative elicitor-regulated elements would provide a plausible "gain" mechanism for very rapid, marked, transient gene activation.

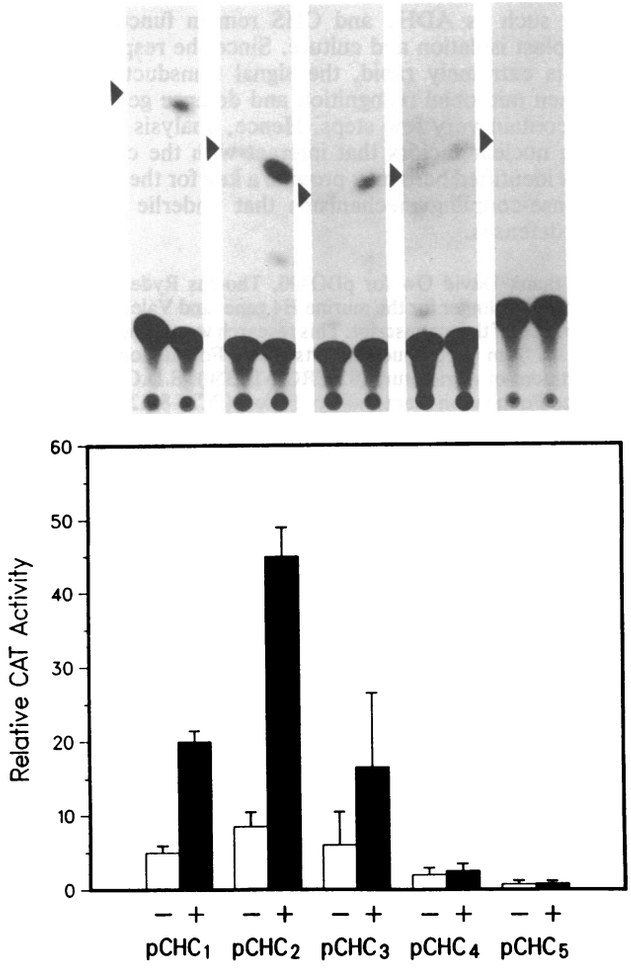

FIG. 5. Effect of 5′ deletions on glutathione regulation of the CHS-CAT-NOS gene electroporated into soybean protoplasts. +, Three hours after addition of glutathione; −, equivalent untreated controls. The structures of 5′ deletions are presented in Fig. 1. Error bars denote standard deviation between independent replicates. Closed arrowheads denote the major CAT product, 3-acetylchloramphenicol.

Two sequence elements, −242 to −194 and −74 to −52, in the 5′ flanking region of CHS (Fig. 1) are strongly conserved in the promoter of a coordinately regulated gene encoding PAL, the first enzyme of phenylpropanoid biosynthesis (C. L. Cramer, K. Edwards, W. Schuch, R.A.D., and C.J.L., unpublished observations). These motifs, which are similarly arranged in the PAL promoter, may therefore have roles in silencer and activator function, respectively. Analysis of point mutations and chimeric promoters will define more precisely the silencer and activator sequence elements and delineate the function of the silencer in elicitor regulation. Previous studies have shown that glutathione and the fungal elicitor have almost identical qualitative effects on the pattern of gene expression and protein synthesis (5). The 5′ deletions examined here have similar effects on regulation by glutathione and fungal elicitor, and it will be of considerable interest to determine, by further dissection of the CHS promoter, whether identical cis-acting elements are involved in transduction of the signal(s) arising from these two different classes of elicitor.

Although expression of genes introduced into protoplasts has been demonstrated in several cases, the only previous report of appropriate regulation in response to an external cue is the stimulation of a chimeric alcohol dehydrogenase 1 (ADH1)-CAT-NOS gene in electroporated maize protoplasts induced by oxygen depletion (28). It appears that the signal transduction mechanisms for activation of stress-induced genes such as ADH1 and CHS remain functional during protoplast isolation and culture. Since the response to elicitors is extremely rapid, the signal transduction pathway between microbial recognition and defense gene activation may contain very few steps. Hence, analysis of the trans-acting nuclear factors that interact with the cis-acting elements identified here may provide a key for the dissection of response-coupling mechanisms that underlie induction of plant defenses.

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