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Combination of H-box [CCTACC(N)$_7$CT] and G-box (CACGTG) cis elements is necessary for feed-forward stimulation of a chalcone synthase promoter by the phenylpropanoid-pathway intermediate *p*-coumaric acid

(flux control/phenylpropanoid natural products/plant gene regulation)

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ABSTRACT **The phenylpropanoid pathway intermediate *p*-coumaric acid (4-CA) stimulates expression of the bean (*Phaseolus vulgaris* L.) chalcone synthase (malonyl-CoA:4-coumaroyl-CoA, EC 2.3.1.74) *chs15* gene promoter in electroporated protoplasts of alfalfa (*Medicago sativa* L.). We have analyzed the effects of 5′ deletions, mutations, and competition with promoter sequences in trans on the expression of a *chs15* promoter–chloramphenicol acetyltransferase gene fusion in elicited alfalfa protoplasts. Two distinct sequence elements, the H-box (consensus CCTACC(N)$_7$CT) and the G-box (CACGTG), are required for stimulation of the *chs15* promoter by 4-CA. Furthermore, a 38-base-pair *chs15* promoter sequence containing both cis elements conferred responsiveness to 4-CA on the cauliflower mosaic virus 35S minimal promoter. The H-box and G-box in combination establish the complex developmental pattern of *chs15* expression and are also involved in stress induction. Hence, potential internal pathway regulation through feed-forward stimulation by 4-CA operates by modulation of the signal pathways for developmental and environmental regulation.**

Plant development and responses to environmental stimuli involve the selective activation of specific sets of genes. Phenylalanine ammonia-lyase (PAL) catalyzes the first step in the central, common pathway of phenylpropanoid biosynthesis, and chalcone synthase (CHS; malonyl-CoA:4-coumaroyl-CoA; EC 2.3.1.74) catalyzes the first step in the branch pathway of phenylpropanoid synthesis specific to the elaboration of flavonoid products (1–3). The expression of *pal* and *chs* genes is under exquisite environmental and developmental control, and specific cis elements in *pal* and *chs* promoters together with their cognate trans factors represent the targets of a number of distinct signal pathways (4–6).

Accumulation of PAL and CHS transcripts in elicitor-treated bean cell-suspension cultures is repressed by the addition of relatively high (10^{-4}–10^{-3} M) concentrations of *trans*-cinnamic acid, the immediate product of the PAL reaction (7, 8), whereas low concentrations increase PAL enzyme activity in elicitor-treated bean cells and excision-wounded epicotyls (9). Reduction of intracellular *trans*-cinnamic acid levels by inhibition of PAL enzyme activity *in vivo* with the specific inhibitor L-α-aminooxy-β-phenylpropionic acid (10) results in a superinduction of PAL and CHS transcripts (8), suggesting that endogenous pathway intermediates function as signal molecules *in vivo*.

To delineate the mechanisms by which pathway intermediates regulate transcript accumulation, we have studied the transient expression of a *chs15* promoter–chloramphenicol acetyltransferase (CAT) reporter gene fusion in electroporated alfalfa protoplasts (11). *chs15* is a member of a family of seven *chs* genes in bean (5, 12). Expression of the *chs15*–CAT gene fusion is repressed by *trans*-cinnamic acid in elicitor-treated alfalfa protoplasts in a manner similar to the resident *chs15* gene in bean-suspension cultured cells. In contrast, *p*-coumaric acid (4-CA), the second intermediate in the pathway, stimulates expression at concentrations as low as 5×10^{-6} M, with a maximum stimulation of 4.5-fold at 5×10^{-4} M. Treatment of pea epicotyl segments with L-α-aminooxy-β-phenylpropionic acid, while superinducing PAL and CHS transcripts in the later stages of the wound response, causes a slower initial induction (V. P. M. Wingate, M. Dron, and C.J.L., unpublished work), consistent with the hypothesis that early accumulation of 4-CA stimulates gene expression before the down-regulation of the system after further build-up of phenylpropanoid metabolites.

By a combination of deletional and mutational analysis together with trans-competition experiments, we now define two specific cis elements, the H-box [CCTACC(N)$_7$CT] and G-box (CACGTG), which are necessary and in combination apparently sufficient for stimulation of expression of the *chs15* promoter by 4-CA. Because these cis elements also play a key role in *chs15* expression in flowers and roots and induction by stress stimuli, our data indicate that potential internal pathway regulation through feed-forward stimulation by 4-CA operates by modulation of signal pathways for developmental and environmental regulation.

MATERIALS AND METHODS

DNA Constructs. All DNA manipulations were done as described in Sambrook *et al.* (13). 5′-Deletions of *chs15* were made by using exonuclease digestion (5), and linker-scanning mutants were constructed by using a Bio-Rad *in vitro* mutagenesis system, following instructions supplied by the manufacturer. p5HB and p5mHB were constructed by hybridizing complementary oligonucleotides synthesized using a DuPont generator DNA synthesizer. Hybridized oligonucleotides were then ligated, and after separation by gel electrophoresis pentamers were cloned into the *Bam*HI site of pIBI25 (Fig. 1*B*).

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Abbreviations: 4-CA, *p*-coumaric acid; CaMV, cauliflower mosaic virus; CAT, chloramphenicol acetyltransferase; CHS, chalcone synthase; PAL, phenylalanine ammonia-lyase.

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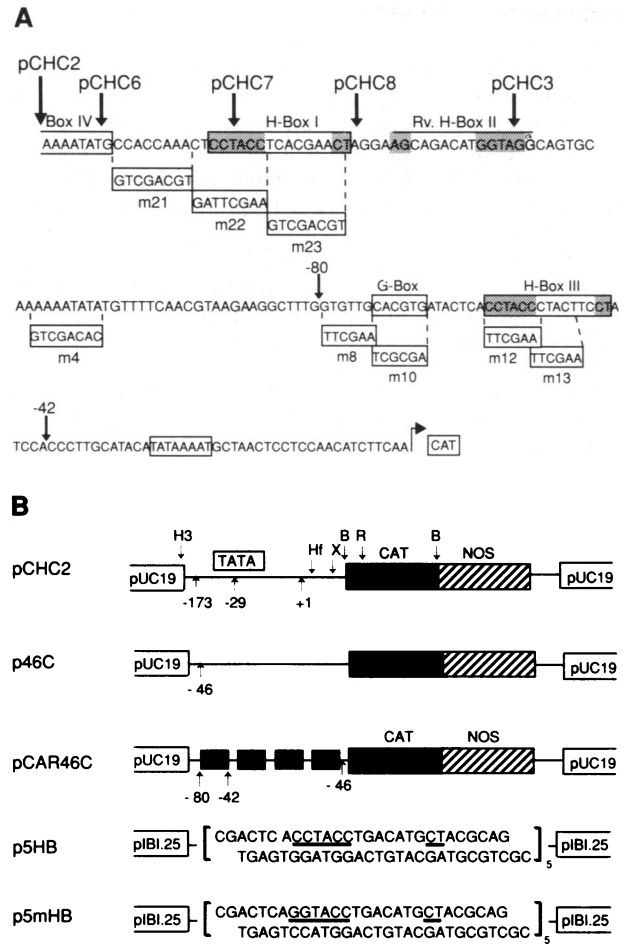

FIG. 1. (*A*) Schematic representation of the *chs15* promoter from position −173 to +1. The *chs15* gene is transcriptionally fused to the CAT reporter gene at position +104. Defined trans-factor binding motifs are boxed. The overscored Rv. H-box II is an H-box sequence in the opposite orientation to H-boxes I and III on the "lower" strand. Shaded areas indicate conserved nucleotides within the three H-box motifs. Boxed sequences below the wild-type *chs15* sequence show the altered nucleotides and their position in each mutant promoter. (*B*) DNA constructs used in the present work (not drawn to scale). pCHC2 contains a transcriptional fusion of the *chs15* promoter from −173 to the CAT reporter gene and nopaline synthase (NOS) terminator in plasmid pUC19 (5). p46C contains a 5′ deletion of the CaMV 35S promoter to −46 (minimal promoter) (14) fused to the CAT gene. pCAR46C contains four copies of the −80 to −42 region of the *chs15* promoter fused to the −46 CaMV minimal promoter. p5HB contains five copies (four head-to-tail, one in opposite orientation) of a 28-bp double-stranded synthetic oligonucleotide containing the H-box CCTACC(N)$_7$CT motif. In p5mHB, the first two cytosine residues of each H-box consensus are changed to guanine residues.

Transient Assays. Protoplasts derived from alfalfa cell suspension cultures (11) were electroporated with the required DNA construct(s) (40 μg), treated with fungal elicitor from the cell walls of *Colletotrichum lindemuthianum* (50 μg equivalents·ml^{-1}) plus or minus 4-CA and collected 8 hr later for assay of CAT activity. In each experiment, protoplasts from the same batch were divided into a number of equal aliquots sufficient for examining the expression of pCHC2 (internal control, Fig. 1*B*) plus the test construct over the full range of 4-CA concentrations tested. Experiments were repeated with different batches of protoplasts, and the data were normalized to the expression value for each construct in the absence of 4-CA (arbitrarily set as 100%). Absolute CAT activities from 307 to 2538·pmol·min^{-1}·mg of protein^{-1} were recorded for the various constructs in the absence of 4-CA. Separate sets of control promoter 4-CA dose–response relationships were plotted for the experiments in Figs. 2–5. The data are presented as the normalized average values ± SDs for replicate independent determinations. In some reports using transient assay systems a constitutive reference gene is coelectroporated with the test construct to compensate for variations in expression seen between experiments (15). That strategy was not used in the present study because of (*i*) the low variability of expression of a given construct within a single batch of protoplasts (11, 16), and (*ii*) the possibility of titrating transcription factors involved in expression of the test construct by a control promoter in trans (17).

RESULTS

5′-Deletion Analysis. Previous 5′-deletion experiments implicated sequences between −173 and −130 of the *chs15* promoter in the stimulation of expression by 4-CA (11). To define more precisely which cis element(s) confer this response, a further set of 5′-deletions with end points between −173 and −130 was constructed (Fig. 1*B*). In these studies the −173 *chs15* promoter–reporter construct (pCHC2) was included in each experiment as a positive control for 4-CA stimulation. Deletion of the *chs15* promoter from position −173 to −166 (construct pCHC6) had no significant effect on stimulation of expression by 5 × 10^{-4} M 4-CA (Fig. 2). This deletion removes all but 2 base pairs (bp) of alfalfa box IV, an *in vitro* DNase I-footprinted region extending from position −177 to −166 (6). In contrast, deletion to position −156 (construct pCHC7) significantly reduced 4-CA stimulation,

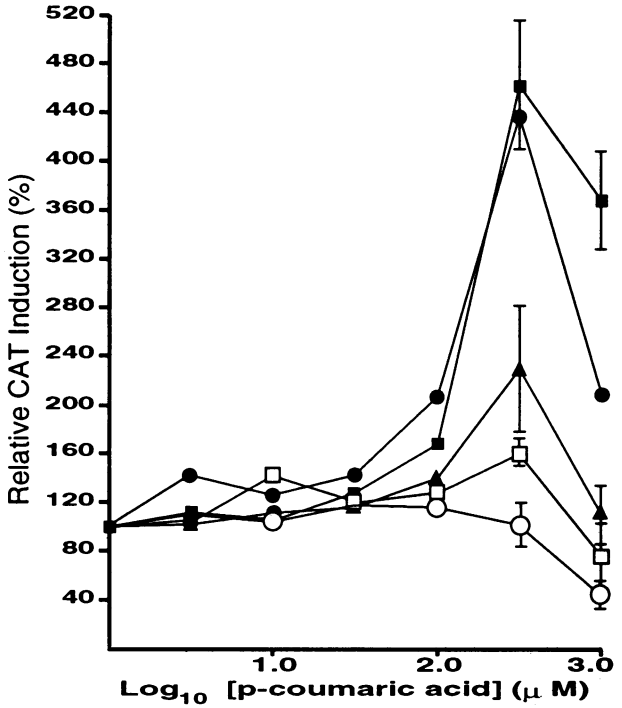

FIG. 2. Dose–response curves for effects of 4-CA on expression of a series of 5′-deleted *chs15* promoter–CAT constructs in electroporated alfalfa protoplasts. ■, pCHC2 (−173 deletion). Test deletions were pCHC6 (−166) (●), pCHC7 (−156) (▲), pCHC8 (−143) (○), and pCHC3 (−130) (□). The pCHC2 control curve here and in Fig. 3 shows the overall averages and SDs of the replicate control dose–response curves done in parallel with the various replications of the individual test constructs. Relative absolute levels of expression (pCHC2:pCHC6:pCHC7:pCHC8:pCHC3) in the absence of 4-CA were 1:3:3.5:3.5:5.

whereas further deletion to position −143 or position −130 (constructs pCHC8 and pCHC3, respectively) almost completely abolished 4-CA stimulation (Fig. 2). Therefore, sequences between positions −166 and −143 appear necessary for stimulation by 4-CA.

It should be noted that the overall expression level of the −173 promoter is affected by deletion (Fig. 2) or mutation (Fig. 3). This presumably results from changes to potential activating or silencing cis elements. Levels of overall expression do not appear related to the responsiveness of the promoter construct to 4-CA.

H-Box Elements Function in 4-CA Stimulation. To date, a single cis element, the H-box [consensus sequence CCTACC(N)$_7$CT], located between −153 and −139 (H-box I) has been defined within the −166 to −130 region (18). H-box sequences are also found at positions −62 to −48 (H-box III) and, in a reverse orientation, between positions −120 and −134 (H-box II). To confirm that H-box I is necessary for 4-CA stimulation and to assess the role of related sequences, a series of linker-scanning mutations was constructed in the context of the −173 *chs15* promoter (Fig. 1*A*). Construct pCHC2m4 possesses a mutation within a region of the *chs15* promoter not associated with any known cis elements. The response of pCHC2m4 to 4-CA was not significantly different from that of pCHC2 (Fig. 3*D*). Therefore, pCHC2m4 effectively acts as a control for the effects of mutations in the H-box and other putative cis elements.

pCHC2m21, which contains a mutation in a functionally undefined A+C-rich sequence, responded to 4-CA with only a slight decrease in stimulation at 5×10^{-4} M (Fig. 3*A*). However, pCHC2m22, in which the CCTACC sequence of H-box I was mutated, was stimulated only 2.1-fold by 4-CA treatment (Fig. 4*B*). This result confirms the conclusion drawn from the 5′-deletion analysis, that H-box I is required for maximum stimulation by 4-CA. Moreover, construct pCHC2m23, in which the (N)$_6$C sequence within H-box I was mutated, showed a similar reduction in responsiveness to 4-CA, exhibiting a 1.8-fold stimulation over control levels of expression (Fig. 3*C*).

To determine whether H-box III is also involved in the 4-CA response, constructs pCHC2m12 and pCHC2m13 were examined. In pCHC2m12, substitution of the CCTACC sequence in H-box III resulted in a decrease in maximum stimulation to 1.5-fold at 5×10^{-4} M 4-CA (Fig. 3*G*). Therefore, H-box III in addition to H-box I is required for maximum 4-CA stimulation. In construct pCHC2m13, the bases C(N)$_5$ in the consensus sequence of H-box III are replaced, and hence only one cytosine residue in the conserved motif is mutated. pCHC2m13 showed no significant difference in 4-CA stimulation when compared with the pCHC2 control (Fig. 3*H*). Therefore, the bases mutated in

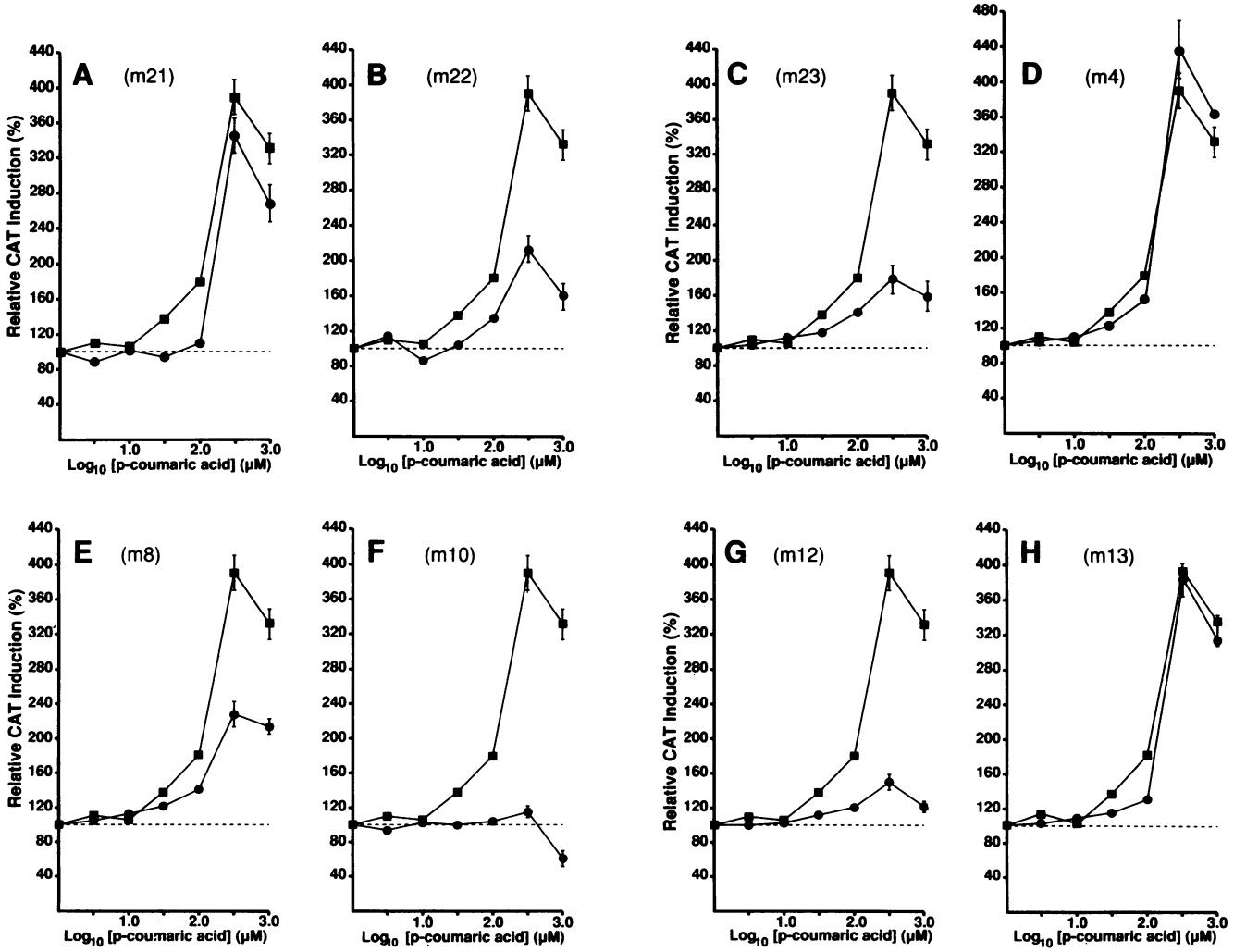

FIG. 3. Dose–response curves for effects of 4-CA on expression of a series of linker-scanning mutants of the −173 *chs15* promoter. (*A–H*) ■ indicates pCHC2 (−173, not mutated). Mutations (●, see Fig. 1) were as follows: pCHC2m21 (*A*), pCHC2m22 (*B*), pCHC2m23 (*C*), pCHC2m4 (*D*), pCHC2m8 (*E*), pCHC2m10 (*F*), pCHC2m12 (*G*), and pCHC2m13 (*H*). Relative expression of these mutant promoters to pCHC2 in the absence of 4-CA was, respectively, 1 (pCHC2):3.5:2:5:0.9:1.1:0.7:0.5:0.9.

pCHC2m13 are not involved in coordinating 4-CA stimulation and are not essential for cognate trans-factor binding to the H-box III motif (compare the effects of the pCHC2m23 mutation in H-box I, Fig. 3C).

G-Box Function in 4-CA Stimulation. Within the proximal region of the *chs15* promoter a distinctive feature is the presence of a G-box, located between positions −75 and −68. The G-box was first identified as a conserved cis element present in the promoters of light-regulated ribulose-bisphosphate carboxylase small subunit (*rbc-S*) genes (19) and has subsequently been implicated in the regulation of a number of plant genes in response to a variety of different signals (20–22). Constructs pCHC2m8 and pCHC2m10 contain mutations in the flanking region immediately 5′ of the G-box core sequence (CACGTG) and within the consensus core-binding sequence of the *chs15* G-box motif, respectively. pCHC2m8 shows a decrease in maximum 4-CA stimulation to 2.3-fold (Fig. 3E), whereas the response of pCHC2m10 to 4-CA was almost completely abolished (Fig. 3F). The G-box motif is, therefore, required for 4-CA stimulation of the *chs15* promoter.

4-CA Regulation of a Heterologous Promoter Fusion. To test whether the H-box and G-box together are sufficient for 4-CA stimulation, we investigated whether multiple copies of the H-box and G-box cis elements would confer responsiveness to 4-CA when placed upstream of the cauliflower mosaic virus (CaMV) 35S minimal promoter. Construct pCAR46C contains a tetramer (head-to-tail fusion) of the *chs15* promoter sequences between positions −80 and −42 (containing both the G-box and H-box III) fused to the minimal (−46) CaMV 35S promoter–CAT reporter construct p46C. Fig. 4 shows that p46C was relatively unaffected by 4-CA, whereas, in contrast, expression of pCAR46C was stimulated 4.5-fold by 5×10^{-4} M 4-CA. Therefore, the H-box and G-box together may be sufficient for 4-CA stimulation, although the possibility that other trans factors may bind within the −80 to −42 *chs15* promoter sequence cannot be completely discounted.

Competition in Trans. To help establish whether the loss of 4-CA stimulation in the *chs15* promoters containing mutant H-box or G-box sequences resulted from the prevention of binding of a cognate trans factor, competition experiments were done in which cis-element multimers were coelectroporated in trans with a wild-type promoter–reporter construct. Coelectroporation of pCHC2 with plasmid p5HB, which contains five copies of the H-box motif cloned in pIBI25, markedly decreased the stimulation of promoter expression by 4-CA. In contrast, coelectroporation of p5mHB, which contains five copies of a mutant H-box sequence that cannot bind the cognate H-box trans factor(s) *in vitro*, had no significant effect in trans on pCHC2 expression (Fig. 5), indicating the involvement of a sequence-specific alfalfa trans factor in the mediation of 4-CA stimulation of the *chs15* promoter. However, similar trans-competition experiments with cloned oligomers of the G-box motif did not significantly reduce 4-CA stimulation (data not shown).

DISCUSSION

Mutant promoter analysis, expression of a heterologous promoter fusion, and trans-competition experiments have demonstrated that two different cis elements within the bean *chs15* promoter, the G-box and H-box, in combination are necessary and apparently sufficient for feed-forward stimulation by the phenylpropanoid-pathway intermediate 4-CA. These data further support the hypothesis that pathway intermediates may act to regulate expression of phenylpropanoid pathway genes, but they do not directly prove a causal involvement of 4-CA. Final proof of such a relation would require genetic dissection. Levels of 4-CA increase ≈4-fold within the first 4 hr after elicitation in alfalfa cells (J. Orr and R.A.D., unpublished results); this increase coincides with the phase of maximum increase in elicitor-induced CHS transcripts. These changes are therefore consistent with a role for 4-CA as a signal molecule during the initial activation of *chs* gene transcription in alfalfa.

The G-box is found only once within the *chs15* promoter, and the almost complete loss of 4-CA stimulation observed with construct pCHC2m10, which contains a mutated G-box, indicates that the G-box has an essential role in 4-CA stimulation. Recent data have revealed a family of genes encoding several distinct factors capable of binding to the

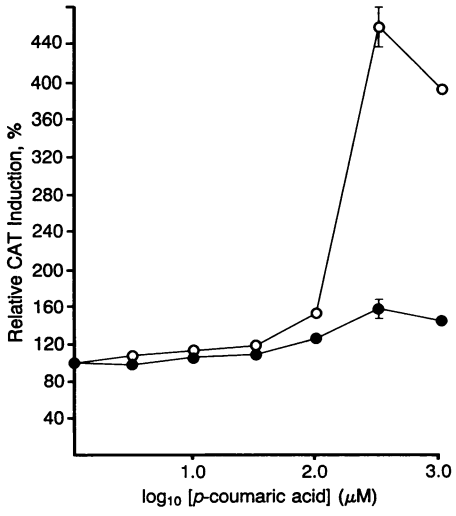

FIG. 4. Effects of *chs15* promoter sequences on 4-CA responsiveness of a minimal CaMV 35S promoter. Dose–response curves are shown for expression of p46C (minimal promoter) (●) and pCAR46C [minimal plus four copies of the −80 to −42 sequence of *chs15* (○)] in electroporated protoplasts. Experimental design was as in Figs. 2, 4, and 5. Relative expression of pCAR46C to that of p46C in the absence of 4-CA was 1:7.8.

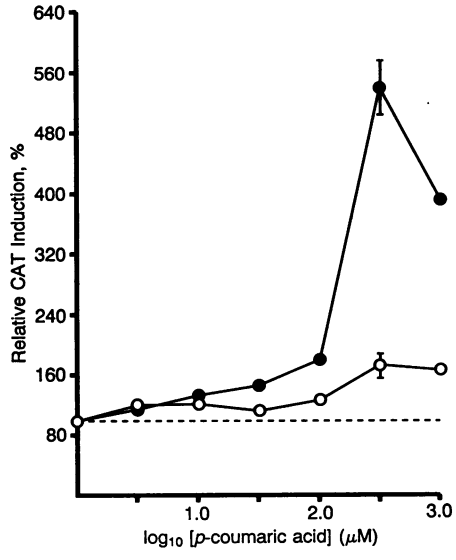

FIG. 5. Effects of coelectroporation of H-box elements on stimulation of expression of pCHC2 by 4-CA. Elicited protoplasts were electroporated with 40 μg of pCHC2 plus 40 μg of p4HB (○) or 40 μg of p4mHB (●) (Fig. 1), treated with a range of 4-CA concentrations and assayed 8 hr later for CAT activity. Expression of pCHC2 plus p4HB relative to that of pCHC2 plus p4mHB in the absence of 4-CA was 1:1.5.

G-box sequence. Therefore, the failure to inhibit 4-CA stimulation by coelectroporation of the G-box in trans might reflect a greater relative abundance of cognate trans factors compared to the H-box trans factor. The specificity of G-box factor binding may be determined by sequences flanking the hexameric G-box core CACGTG (23); this might explain the reduction in 4-CA stimulation observed on mutating the six nucleotides immediately 5′ of the core.

Functional analysis has implicated the G-box in promoter activation by various signals including white light, abscisic acid, and UV light (20–22). However, in the parsley *chs* gene, another motif, box I, in addition to the G-box is required for UV induction (24). Likewise, the G-box is thought to interact with GT-1 (GGTTAA) and I-box (GATAAG) motifs in coordinating light-regulated expression of the *rbc-SA* gene (20), and a tetramer of the G-box alone is not sufficient for abscisic acid-inducible expression of a β-glucuronidase reporter gene in transgenic tobacco (25). For 4-CA stimulation of the *chs15* promoter our data indicate that the G-box functions in combination with H-box sequences. G-box factors are members of the class of basic leucine-zipper transcription factors (26), and hence combinatorial interactions between the two cis elements might reflect direct interactions between G-box and H-box trans factors. In contrast to the single G-box, there are three copies of the consensus H-box sequence CCTACC(N)$_7$CT. Mutation of either H-box III, which is immediately downstream of the G-box (−62 to −48) or the upstream H-box I (−154 to −140), reduced 4-CA stimulation to the near-background levels seen with other plant-defense gene promoters (11). Therefore, H-boxes I and III do not appear functionally redundant with respect to 4-CA stimulation within the context of the natural promoter. For construct pCAR46C, which contains four tandem copies of the −80 to −42 region, one or more of the distal copies of H-box I presumably compensate for the absence of H-box III. In contrast, the loss of 4-CA stimulation by 5′ deletion to −143 or by mutation of H-box I within the context of the −173 promoter indicates that the H-box in the reverse orientation between −135 and −121 (H-box II) cannot compensate for H-box I.

The H-box is also present in the parsley *chs* and *pal* promoters, and functional analysis indicates that this cis element is involved in UV induction (22, 27). Moreover, analysis of the *chs15* promoter in transgenic tobacco plants has shown that mutation of either H-box I or III, or the G-box, resulted in a striking decrease in floral expression and a significant decrease in root expression (O.F., J. M. Kooter, R.A.D., and C.J.L., unpublished work). Furthermore, a tetramer of *chs15* promoter sequences between positions −80 and −42 containing the G-box and H-box III, when fused to the minimal CaMV 35S promoter and β-glucuronidase reporter gene, directed the wild-type developmental expression pattern observed with the full *chs15* promoter and was also induced in leaves after tobacco mosaic virus infection (O.F., G.J.L., R.A.D., and C.J.L., unpublished work). Therefore, the H-box and G-box in combination also play a central role in coordinating the expression of the *chs15* gene in response to developmental and environmental cues.

The combinatorial specificity conferred by the interaction between G-box and H-box cis elements establishes a unique pattern of promoter activity not observed with promoters containing only one of these two elements—e.g., *rbc-S* and *Em*. Because developmental and environmental stimuli use the same pair of cis elements required for 4-CA stimulation, this combinatorial specificity may provide a mechanism to restrict promoter modulation by perturbations in flux in the phenylpropanoid pathway to a specific set of genes with a characteristic pattern of developmental and environmental regulation.

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