Functional characterization of cis-acting elements mediating flavone-inducible expression of CYP321A1

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How plant allelochemicals elicit herbivore counterdefense genes remains largely unknown. To define the cis-acting elements for flavone inducibility of the allelochemical-metabolizing CYP321A1 from Helicoverpa zea, functions of varying length of CYP321A1 promoter are examined in H. zea fatbody cells. Progressive 3'-deletions reveal presence of positive elements in the 5' untranslated region (UTR). Progressive 5'-deletions map out regions of one essential element, four enhancers, and two silencers. Further progressive 5'-deletions localize the essential element to a 36-bp region from 109 to 74. This essential element, designated as xenobiotic response element to flavone (XRE-Fla), contains a 5' AT-only TAAT inverted repeat, a GCT mirror repeat and a 3' antioxidant response element-like element. Internal deletions and substitution mutations show that the TAAT repeat is only necessary for the maximal flavone inducibility, whereas the other two components are necessary for the basal and flavone-induced expression of CYP321A1. Electrophoresis mobility shift assays demonstrate that XRE-Fla specifically binds to H. zea fatbody cell nuclear extracts and flavone treatment increases the nuclear concentrations of the yet-to-be characterized transcription factors binding to XRE-Fla. Taken together, CYP321A1 expression is regulated primarily by XRE-Fla and secondarily by other cis elements scattered in its promoter and 5' UTR.

1. Introduction

Cytochrome P450 monoxygenases (P450s) are a superfamily of heme-binding enzymes that play a paramount role in mediating plant-insect interactions. Plants use P450s to synthesize toxic defensive compounds known as allelochemicals to defend themselves against herbivorous insects, whereas insect herbivores employ P450s to detoxify plant allelochemicals they encounter in their host plants (Schuler, 1996; Berenbaum, 2002). To defer the cost of counterdefense against plant defenses, herbivorous insects have evolved a complex of regulatory machinery, enabling substantial increases of their allelochemical-metabolizing P450s upon exposure to allelochemicals (Berenbaum, 2002; Li et al., 2000, 2002, 2007, 2001; Snyder et al., 1995; Stevens et al., 2000; Danielson et al., 1997; Wen et al., 2009; Petersen et al., 2001). Such allelochemical inducibility is essential for insect herbivores to cope with a diversity of allelochemicals idiosyncratically distributed among potential host plants (Li et al., 2007).

The regulatory cascades that provide allelochemical inducibility still remain largely a mystery. Only two allelochemical regulatory cascades have been studied. One is the caffeine cascade mediating upregulation of CYP6A8 and CYP6A2 in Drosophila melanogaster (Bhaskara et al., 2006, 2008), which is not an herbivore but a saprophagous insect breeding in decaying plant tissues. The other is the xanthotoxin cascade mediating the upregulation of CYP6B1 in Papilio polyxenes, a specialist herbivore, and CYP6B4 in P. glaucus, a generalist herbivore (Brown et al., 2004, 2005; McDonnell et al., 2004; Petersen et al., 2003). While not acting as a plant defense against D. melanogaster, caffeine induces the expression of CYP6A8 and CYP6A2 by inhibiting the cAMP-phosphodiesterase and decreasing the overall levels of D-jun, a transcriptional repressor of the two CYP6A genes (Bhaskara et al., 2006, 2008). How cAMP increase resulted from inhibition of the cAMP-phosphodiesterase by caffeine stimulates CYP6A expression remains unknown. Also unknown are the...
cis-elements for D-jun and other unidentified transcription factors. For the xanthotoxin cascad e, an overlapping edysone response element (EcRE)/antioxidant response element (ARE) / xenobiotic response element to xanthotoxin (XRE-xan) and a xenobiotic response element to aryl hydrocarbon receptor (XRE-AhR) have been functionally characterized from both of the CYP6B1 and CYP6B4 promoters in heterologous Sf9 cells (Brown et al., 2004, 2005; McDonnell et al., 2004; Petersen et al., 2003). Spineline (Ss) and Tango (Tgo), the D. melanogaster homologues of mammalian AhR and aryl hydrocarbon receptor nuclear translocator (ARNT) that bind to XRE-AhR, enhance the basal expression of the CYP6B1 promoter but not the magnitude of its xanthotoxin and benzo[a]pyrene induction (Brown et al., 2005). Other components of the xanthotoxin regulatory cascade including transcription factors have yet to be elucidated. Other than the two aforementioned allelochemical cascades, the bHLH-PAS transcription factors Methoprene-tolerant (Met) and Germ-cell expressed (Gce) may be involved in perception and transduction of juvenile hormone (JH)-like allelochemicals since the dimerized Met-Gce is increasingly believed to act as a JH receptor (Baumann et al., 2010).

To advance understanding of allelochemical regulatory cascades, we choose to study the regulatory control of the allelochemical- and insecticide-metabolizing CYP321A1 (Sasabe et al., 2004; Niu et al., 2008; Wen et al., 2009; Rupasinghe et al., 2007) from Helicoverpa zea, a broadly polyphagous insects with over 100 known host plants (Kogan et al., 1978). CYP321A1 transcripts are constitutively low but highly inducible by allelochemicals, particularly the flavonoid f lavone (Sasabe et al., 2004; Zeng et al., 2007; Niu et al., 2008; Wen et al., 2009), a flavone aglycone present in a wide range of host plant families (Li et al., 2002). Here, we present the basal and flavone-induced luciferase activities of CYP321A1 promoter progressive deletion, internal deletion and substitution mutant constructs in homologous H. zea fat body cells. We also present documentation of specific DNA-protein complexes formed between the 36-bp essential cis element XRE-F1a characterized in this study and H. zea fatbody cell nuclear proteins. These data demonstrate that the basal and flavone-inducible expression of CYP321A1 is regulated by at least eight cis-acting elements including the essential element XRE-F1a.

2. Materials and methods

2.1. Cell culture and analysis of CYP321A1 expression in H. zea fat body cells

The homologous H. zea fat body cell line BCIRL-HzF833 (Goodman et al., 2004), generously provided by Dr. Cynthia L. Goodman (BCIRL, USDA, ARS), was routinely maintained with Ex cell 420 insect serum-free medium (SAFC Biosciences, Lenexa, KS) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone-QB perbio, Logan, UT), 50 U/ml penicillin, 50 μg/ml streptomycin, and 12 μg/ml gentamycin (Invitrogen, CA) in an incubator at 28 °C. To confirm the flavone induction of CYP321A1 in this cell line, cells seeded onto a 6-well plate (9 × 10^6 cells/well) containing 2 ml supplemented medium were treated with methanol (control) or flavone (dissolved in methanol) at a final concentration of 18.5 μM for 48 h. The concentration and incubation time were determined according to Petersen et al. (2003) and our preliminary experiments (data not shown). Total RNA was isolated from these cells using the guanidinium-HCl procedure (Sambrook et al., 1989). Two micrograms of each RNA sample were employed as template for synthesis of the first-strand cDNA with oligo-dT14 primer and M-MLV Reverse Transcriptase (Biolas, MA). One microliter of cDNA was used as template to amplify CYP321A1 and EF-1α (as a reference) transcripts in two separate PCR tubes using the primer pairs 321A1-F/321A1-R and EF-1α-F/EF-1α-R (supplementary Table S1), respectively. The PCR cycling conditions for both genes were denaturation for 4 min at 94 °C, followed by 25 (for EF-1α) or 35 (for CYP321A1) cycles of 1-min denaturation at 94 °C, 1-min annealing at 60 °C, 2-min extension at 72 °C, and a final 10-min extension at 72 °C. RT-PCR products of the two genes from each cDNA template were loaded on the same lanes and analyzed on 1% agarose gels stained with ethidium bromide.

2.2. Construction of CYP321A1 promoter-pGL3 constructs

PCR was employed to generate the following four types of CYP321A1 promoter reporter gene constructs: progressive 5′ deletion-pGL3 constructs, progressive 3′ deletion-pGL3 constructs, internal deletion-pGL3 constructs, and multiple base pair substitution mutation-pGL3 constructs. The common template for progressive 5′ and 3′ deletion-pGL3 constructs was CYP321AID-6-pGL3 (Fig. 1), which was made by subcloning the 3′-most 3995-bp fragment of CYP321AID-6, a 5498 bp 5′-flanking sequence of CYP321A1 obtained previously by genome walking (GenBank accession no: DQ788491, Chen and Li, 2007), into the pGL3-Basic vector (Promega, WI) from the CYP321AID-6-pGEM-T plasmid (Fig. 1). The restriction sites used for subcloning the CYP321A1D0L6 deletion constructs were Mlu I and Xho I. The 3995-bp fragment being subcloned into pGL3 basic vector contains the first 29 bp CYP321A1 coding sequence, 5′ UTR (64 bp), full-length promoter sequence (1470 bp), and the upstream CYP321A2 coding region and part of its 5′ UTR (Fig. 1). Throughout this paper, we report nucleotide position in the proximal promoter relative to the transcriptional start site indicated by +1, with upstream of it preceded by “−” and downstream preceded by “+.”

Progressive 5′ deletion fragments for 5′ deletion constructs were PCR-amplified by using the common reverse primer 321A1-R(+64) and each of the forward primers 321A1-F(−1470), 321A1-F(−1218), 321A1-F(−888), 321A1-F(−558), 321A1-F(−310), 321A1-F(−278), 321A1-F(−237), 321A1-F(−199), 321A1-F(−159), 321A1-F(−109), 321A1-F(−99), 321A1-F(−92), 321A1-F(−79), 321A1-F(−48) (see supplementary Table S1 for their sequences). PCR amplifications were carried out in a 50 μl mixture containing 5 μl of 10 × PCR buffer, 1 μl of 10 mM dNTP, 20 pmol of specific primer pairs, 0.2 μg of the template CYP321A1D16-pGL3, 2.5 μl of Taq DNA polymerase (Lucigen, WI) and 0.25 U of Pfu DNA polymerase (Stratagene, CA). The reason for combinational use of Taq and Pfu DNA polymerases is to take advantage of Pfu’s high fidelity and Taq’s high efficiency. The PCR cycling conditions were as follows: 5-min initial denaturation at 94 °C, followed by 30 cycles of 1-min denaturation at 94 °C, 1-min annealing at 68 °C, 1- or 2-min extension at 72 °C, and a 10-min final extension at 72 °C. Progressive 3′ deletion fragments for 3′ deletion constructs, on the other hand, were PCR-amplified using the common forward primer 321A1-F(−1470) and either of the reverse primers 321A1-R(−84) and 321A1-R(−1) (supplementary Table S1). The PCR set up and cycling conditions for amplifications of the progressive 3′ deletion fragments were the same with those for progressive 5′ deletion fragments except for annealing at 65 °C instead of 68 °C. All the reverse primers were engineered with an Xho I restriction enzyme site, whereas all the forward primers were engineered with a Mlu I restriction enzyme site. PCR products were run on a 1% agarose gel in 1 × TAE buffer. The resultant band for each deletion fragment was eluted from the gel using the QIAquick Gel Extraction Kit (Qiagen, CA), directly cloned into the Xho I and Mlu I sites of the firefly luciferase reporter vector pGL3-Basic (Promega, WI).

Eight internal deletion constructs including P(−1470/+64) HzIS1-3-del, P(−888/+64)M2-del, P(−888/+64)M4-del, P(−888/
Fig. 1. Schematic construction of the CYP321A1DL6-pGL3 construct. The 3’ most 3995-bp fragment of the 5498 bp 5’-flanking sequence of CYP321A1 (named as CYP321A1DL6) was PCR-amplified from the CYP321A1DL6-pGEM-T plasmid using the primers 321A1DL6-F and 321A1DL6-R (see Table S1 for their sequence) and then directly cloned into the Xho I and Mlu I sites of the pGEM-T basic vector. The two inserts in the pGEM-T and pGL3 basic vectors are shown above the two vectors and drawn to scale, with the coding sequence, 5’ UTR, 3’ UTR, and promoter (or 5’ flanking sequence) of CYP321A1 and CYP321A2 indicated. The transposons HzSINE1 and HzSINE1 are depicted as a gray and a black inverted triangle, respectively.

- PCR amplification of CYP321A1DL6-pGL3 constructs

The PCR amplification conditions of the two constructs were as follows: 5-min initial denaturation at 94°C, followed by 28 cycles of 30-s denaturation at 94°C, 1-min annealing/extension at 68°C, and a 10-min final extension at 72°C. The PCR cycling conditions for the third construct were as follows: 5-min initial denaturation at 94°C, 1-min annealing at 58°C, and a final extension at 72°C.

- Substitution mutation construction

The substitution mutation construct P(−888/+64)M3-mut was PCR-cloning into the Xho I and Mlu I sites of the firefly luciferase reporter vector pGL3-Basic. The PCR setup and cycling conditions were the same with those of the first two PCR reactions for the constructs P(−888/+64)M3-mut and P(−888/+64)M4-mut. All the constructs were verified by sequencing in both directions using the forward RVprimer3 and the reverse G primer2, respectively, at the Genomic Analysis & Technology Core Facility of the University of Arizona.

- Transient transfection and dual luciferase assay

H. zea fatbody cells seeded onto a 12-well plate (9 × 10² cells/well) were transiently co-transfected with CYP321A1 promoter-pGL3 luciferase reporter constructs (1.5 or 2 µg/well) and the internal renilla luciferase control reporter plasmid phRL-TK (Promega; 0.15 or 0.2 µg/well) using Cellfectin-II reagent (Invitrogen; 8 µl per well). Sixteen hours post-transfection, flavone at
a final concentration of 18.5 μM (induction) or equal volume of methanol (control) was added. After 48 h, the cells were harvested and the resulting lysates were used to measure the renilla and firefly luciferase activities with the Dual-Luciferase Reporter Assay System (Promega) on a Turner Designs TD-20/20 Single-Tube luminometer (Turner Biosystems, CA). The relative firefly luciferase activity normalized against the renilla luciferase activity reported for each construct represent the mean ± the standard error of three or four independent transfections of one representative experiment. The induction folds reported are expressed as a ratio of the normalized flavone-induced firefly luciferase activity to the normalized basal firefly luciferase activity (methanol control). All experiments were repeated 2–3 times and one representative experiment of 3–4 independent transfections was shown in each figure. Significant differences among constructs of a transfection series were determined by one-way analysis of variance (ANOVA) followed by Tukey’s HSD tests for multiple comparisons.

2.4. Electrophoretic gel mobility shift assay

H. zea fatbody cells seeded onto a 6-well plate (9 × 10⁵ cells/well) were treated with 18.5 μM of flavone (dissolved in methanol) or equal volume of methanol (control). After 48 h, the cells were harvested and used to prepare un-induced (methanol) and flavone-induced H. zea fatbody cell nuclear extracts according to Lahiri and Ge (2000). Biotin end-labeled double-stranded oligonucleotides (5′-agagATAATATACGGCTGTAACAGTTCGCCACAAAcca-biotin-3′, the fragment between –113 to –71 of CYP321A1 promoter) containing the essential XRE-Fla (the capital letters in the sequence) were used as the probe in gel shift experiments. DNA-nuclear extracts binding reactions were carried out in a 20 μl mixture containing 0.17 μg of H. zea fatbody cell nuclear extracts, 2 μl of 10 × binding buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 1 μl of poly(dI-dC) (1 μg/μl) and 80 fmol of the biotin-labeled probe. The reaction mixture was preincubated for 10 min at 25 °C, after which the probe was added. Incubation continued for another 20 min at 25 °C. Competition reactions were done in the same conditions. Only the difference was the addition of 200-fold molar excess of the unlabelled competitor oligonucleotides M (–109 to –74, i.e. the cold probe), M4 (–92 to –74), M3 (–99 to –93), M2 (–109 to –100) [each contains 3 or 4 flanking nucleotides at both ends; see Fig. 3 and supplementary Table S1 for their sequences] or the unrelated nonspecific oligonucleotide C (see Table S1 for its sequence). The reaction mixtures were electrophoresed through a pre-run (1–1.5 h) 4–20% non-denaturing polyacrylamide gradient gel (Bio-Rad) for 3.5 h in 0.5 × TBE buffer at 70 volts at 4 °C, and then electro-transferred onto a Hybond-N nylon membrane (Amersham Biosciences) for 50 min at 220 mA at 4 °C. The retarded DNA-nuclear protein complexes on the membrane were fixed by UV cross-linking (twice of 1 min each at 120 mj/cm²) and visualized with a LightShift Chemiluminescent Electrophoretic Mobility Shift Assay Kit (Pierce). The intensities of the sequence-specific bands formed were quantified using the ImageJ software.

3. Results

3.1. CYP321A1 transcription in H. zea fatbody cell

To assess the suitability of the H. zea fatbody cell line BCIRL-HzFB33 (Goodman et al., 2004) for functional characterization of cis-acting elements and transcription factors that regulate the basal and flavone-inducible expression of CYP321A1, we conducted RT-PCR gel analyses with mRNA samples extracted from control and flavone-induced cells. Consistent with previous studies on larval tissues (Wen et al., 2009; Niu et al., 2008; Zeng et al., 2007), expression of CYP321A1 in the fatbody cell line was constitutively low but highly inducible by flavone (Fig. 2). This result confirms that the H. zea fatbody cell line has the transcriptional and signaling machinery necessary for basal and flavone-inducible expression of CYP321A1.

3.2. Putative cis elements within the CYP321A1 promoter and 5′ untranslated region

We previously cloned a 5498-bp 5′-flanking sequence of CYP321A1 from a laboratory strain of H. zea by genome walking (GenBank accession no. DQ788841). This 5498 bp 5′-flanking sequence contains its promoter sequence and CYP321A2, a paralog of CYP321A1 (Chen and Li, 2007). The wild type CYP321A1 promoter sequence, i.e. the intergenic region between the two P450 paralogs, is 1470-bp long and contains a transposon named HzIS1−3 from –1348 to –1149 (Fig. 3; Chen and Li, 2007). In silico screening of the CYP321A1 promoter and 5′ untranslated region (UTR) sequences for cis-elements by using Transcription Element Search Software (TESS) (http://www.cbil.upenn.edu/tess) and manual search revealed multiple putative elements sharing at least 85% sequence similarity with those in other regulated vertebrate and insect promoters. These include one ERE, two ARE (Wasserman and Fahl, 1997), one XRE-AhR, three octamer transcription factor-1 (Oct-1) site (Sterling and Bresnick, 1996; Pitarque et al., 2005; Thum and Borlak, 2004, 2008), and three arthropod initiator elements (Inr) (Cherbas and Cherbas, 1993) (Fig. 3). Although these putative elements may or may not be functional elements in the CYP321A1 promoter, we considered their positions when we made progressive and internal deletion constructs.

3.3. Roles of 5′UTR in the transcriptional regulation of CYP321A1

To determine whether 5′UTR is important for the basal and/or flavone-induced expression of CYP321A1, we generated a set of three CYP321A1 promoter 3′ progressive deletion constructs from nucleotide +84 to –1 relative to the transcription start site (Fig. 3 for the sequence of each construct). All the three deletion constructs had significant basal and flavone-induced luciferase activity compared with the promoterless pGL3-basic construct (Fig. 4A). The basal promoter activity of the promoter plus 5′UTR construct P(–1470/+64) was 8.06- and 1.82-fold greater than that of the promoter only construct P(–1470/–1) and the coding

![Fig. 2. CYP321A1 expression in homologous H. zea fatbody cells. Homologous H. zea fatbody cells seeded onto a 6-well plate at 9 × 10⁵ cells/well were induced by 18.5 μM flavone or equal volume of methanol (control) for 48 h. Total RNA extracted from these cells were used as templates to amplify CYP321A1 and elongation factor 1-α (EF-1α, reference gene) transcripts by RT-PCR gel analysis detailed in Materials and methods. RT-PCR products of the two genes from each cDNA template were loaded on the same lanes and analyzed on 1% agarose gels stained with ethidium bromide. Shown in this figure is a representative gel picture.](http://example.com/fig2.png)
sequence-containing construct P(−1470/+84), respectively. The flavone-induced luciferase activity of P(−1470/+64) was 5.93- and 1.69-fold greater than that of P(−1470/+1) and P(−1470/+84), respectively. Because P(−1470/+64) exhibited greater increase in the basal activity than in the flavone-induced activity, its flavone inducibility (9.5 fold) was actually lower than those of P(−1470/+84) (10.25 fold) and P(−1470/+1) (12.92 fold) (Fig. 4A). The results suggest that while CYP321A1 promoter alone is capable of regulating the basal and flavone-induced expression of this gene, the maximal basal and flavone-induced expression of CYP321A1 necessitates its 5′UTR sequence but not its coding sequence. The results also suggest that the 20-bp coding sequence (+65 to +84) repress basal and flavone-induced promoter activity.

3.4. Roles of HzIS1-3 in the transcriptional regulation of CYP321A1

A CYP321A1 promoter internal deletion construct named P(−1470/+64)HzIS1-3-del that deletes the transposable element HzIS1-3 and its left target site duplicate (TSD) (−1348 to −1154, Fig. 3) was generated to address the question of whether HzIS1-3 affects the transcription of CYP321A1. Compared with the promoter plus 5′UTR construct P(−1470/+64), deletion of HzIS1-3 increased the basal (29.3%) and flavone-induced (36.0%) promoter activity as well as the induction fold, but none of these increases were statistically significant (Fig. 4B). These data suggest that HzIS1-3 insertion slightly repress the basal and flavone-induced expression of CYP321A1.
3.5. Localization of cis-acting elements by progressive 5′ deletions

In order to roughly define the regions harboring various cis elements, a set of eight CYP321A1 promoter 5′ progressive deletion constructs (P(−1470/+64), P(−1218/+64), P(−888/+64), P(−558/+64), P(−310/+64), P(−278/+64), P(−79/+64), P(−48/+64)) (see Fig. 3 for their boundaries) were co-transfected into H. zea fatbody cells with phRL-TK control plasmid. Relative to the promoter plus 5′ UTR construct P(−1470/+64), progressive 5′ deletions to −1218, −888, and −558 significantly increased the basal promoter activity, whereas 5′ deletion to −79 and −48 significantly reduced or abolished [P(−48/+64)] it (Fig. 4C). 5′ deletion to −310 marginally increased it (by 34%, not significant), whereas 5′ deletion to −278 slightly reduced it (by 45%, not significant).

A similar trend was observed for the flavone-induced activity. Compared to P(−1470/+64), progressive 5′ deletions to −1218 and −888 significantly enhanced the flavone-induced activity, whereas 5′ deletions to −79 and −48 completely blocked it (Fig. 4C). 5′ deletions to −558 and −310 increased it by 40.0% and 34.9% (not significant at P < 0.05), respectively, whereas 5′ deletion to −278 reduced it by 23.1% (not significant). Because of the aforementioned increases and reductions in both the basal and flavone-induced activities, flavone inducibility (i.e. induction fold) varied significantly among the eight 5′ deletion constructs. Multiple comparison tests on the induction fold divided the eight 5′ deletion constructs and the promoterless pGL3-basic construct into 3
significantly different groups. P (−1218/+64) and P (−278/+64) had the highest induction fold (>13 fold), P (−79/+64), P (−48/+64) and pGL3-basic had the lowest induction fold (1.2−1.3 fold, no significant induction), and the remaining deletion constructs fell between the two other groups (8.95 to 11.68 fold) (Fig. 4C). These results indicate that there is one enhancer located between −278 to −310 and −558 to −888, respectively, and one silencer between −1218 to −1470. However, the essential cis element required for both the basal and flavone-induced expression of CYP23A1, designated as xenobiotic response element to flavone (XRE-Fla), is located in the 200-bp region between −278 to −79.

3.6. Fine mapping of XRE-Fla, the CYP23A1 essential cis element

To further narrow down the essential XRE-Fla, we generated a set of 4 progressive 5′ deletion constructs from −278 to −79; each deletes about 40 bp from its adjacent 5′ construct. Compared to P (−278/+64), deletions to −237, −199, and −195 resulted in 31.1% (not significant), 221.2% (significant) and 107.9% increases (significant) in the basal activity and 19.3% (not significant), 42% (significant), and 56.4% (significant) increases in the flavone-induced promoter activity, respectively (Fig. 4D). By contrast, deletions to −119 and −79 caused 9.2% (not significant) and 67.4% (significant) reductions in the basal activity and 1.17% (not significant) and 98.5% (significant) reductions in the flavone-induced activity, respectively (Fig. 4D). Multiple comparison tests revealed that P (−278/+64), P (−237/+64), and P (−119/+64) produced the highest induction fold (>30 fold), followed by P (−159/+64) (24.63 fold), P (−195/+64) (14.47 fold), and P (−79/+64) (1.5 fold, basically no induction). These data indicate that both the regions from −119 to −159 and from −159 to −195 contain a major positive cis-element, whereas the region from −199 to −237 has a major negative cis-element. The XRE-Fla is located from −119 to −79 or a few bp downstream of −79.

Manual examination of the −119 to −74 sequence found that it starts with a direct repeat of “GACA” motif (−116 to −109), followed by an inverted repeat of “TAAT” motif spaced by one deoxyadenosine (−109 to −100), a mirror repeat of “GCT” motif (−98 to −93), and the final 19-bp fragment (−92 to −74) (Fig. 4E). The final 19-bp fragment is called ARE-like element because it resembles the ARE consensus sequence 5′-TAANNRTGAYnnnGCTWwwww-3′ (Wasserman and Fahl, 1997) found in the promoters of Phase II detoxification genes in mammals. To determine which of the above four repeats or motifs comprise the XRE-Fla, three more 5′ deletion constructs [P (−109/+64), P (−95/+64) and P (−92/+64)] that progressively deletes the beginning GACA direct repeat (M1 in Fig. 4E), the TAAT inverted repeat (M2) and the GCT mirror repeat (M3) were generated. These were co-transfected into H. zea fatbody cells with pHRL-TK control plasmid. Compared to the construct P (−119/+64), deletion to −109 had little effect on the basal and flavone-induced activities (Fig. 4E), indicating that the GACA direct repeat is not a component of the essential element XRE-Fla. By contrast, deletion to −99 completely blocked the flavone inducibility while increasing the basal activity by 47.7% (significant at P < 0.05). Further deletions to −92 and −79 not only completely abrogated the flavone inducibility, but also reduced the basal activity by about 48% (significant at P < 0.05). These results suggest that the XRE-Fla is 36-bp long (from −109 to −74) and is composed of the TAAT inverted repeat, the GCT mirror repeat and the ARE-like element.

To further determine the roles of each component, we generated a set of internal deletion constructs that delete any one of the three components or the whole XRE-Fla element from the short construct P (−278/+64) and/or the long construct P (−888/+64), respectively. Deletion of the TAAT inverted repeat (M2 in Fig. 5A) significantly reduced the flavone-induced activity and flavone inducibility in both the short and long constructs, but increased the basal activity by 24.7% (significant) in the short construct while reducing it by 42.4% (significant) in the long construct (Fig. 5B and C). Deletion of the GCT mirror repeat (M3) from the short construct caused a 54.6% reduction in the basal activity and a 90.8% reduction in the flavone-induced activity, leaving only a marginal level of flavone inducibility (1.90 fold) (Fig. 5D). Deletion of the ARE-like element (M4) from both the short and long constructs resulted in the largest reductions of the basal and flavone-induced activities and the complete knockout of flavone inducibility (<1.4 fold) (Fig. 5E and F). The resultant reductions in the basal and flavone-induced activities from deletion of the ARE-like element were even larger than (although not significant) those from deletion of the whole XRE-Fla element (Fig. 5E and F). Taken together, the ARE-like element and the GCT mirror repeat are necessary for both the basal and flavone-induced expression, whereas the TAAT inverted repeat is necessary for the flavone inducibility but not for the basal expression.

3.7. Mutational analysis of XRE-Fla

To characterize the necessary nucleotides within the XRE-Fla, we constructed three multiple base pair substitution and mutation constructs (Table S1) (Fig. 5I). Each altering one nucleotide within the TAAT inverted repeat, the GCT mirror repeat, or the ARE-like element (Fig. 5A). Mutation of the TAAT inverted repeat TAATTAAT to AgggccTA, which not only disrupts the inverted repeat but also shifts it from a AT only repeat to a GC rich sequence, resulted in a 90.2% reduction in the flavone-induced activity but only a 3.1% reduction in the basal activity (Fig. 5G). Such effects were consistent with those exhibited by the corresponding internal deletion constructs (Fig. 5B and C). Mutation of the GCT mirror repeat CGCTTGC to CgaccAG, which changes its 4 central nucleotides while keeping its mirror repeat structure, reduced the flavone-induced activity by 61.3% but increased the basal activity by 55% (Fig. 5H). For comparison, internal deletion of this repeat significantly decreased both the basal (54.6%) and the flavone-induced (90.8%) activity (Fig. 5D). These comparisons suggest that the unmutated 5′ GC dinucleotide, the 3′ G, and the mirror repeat are probably more essential than the 4 central nucleotides, especially for the basal activity. Mutation of the ARE-like element TAAACATGATTTGCCGAAA to TAAACagGTCTTAgAAA, like deletion of the whole ARE-like element (Fig. 5E and F), resulted in the largest reductions in the basal and flavone-induced activities and complete knockout of flavone inducibility (Fig. 5I). This indicates that the altered nucleotides TGAT and GC are essential for the function of the ARE-like element.

3.8. H. zea fatbody cell nuclear proteins bind to the XRE-Fla

To determine whether the essential element XRE-Fla is recognized and specifically bound by the nuclear extracts prepared from un-induced (methanol treated) and/or flavone-induced H. zea fat-body cells, we conducted electrophoretic mobility shift assays (EMSA) (Fig. 6). We used the biotin-labeled double-stranded oligos containing the XRE-Fla (see Table S1 for its sequence) as the probe and the unlabeled XRE-Fla (i.e. cold probe, M in Fig. 6). TAAT inverted repeat (M2), GCT mirror repeat (M3), ARE-like element (M4), and an unlabeled unrelated nonspecific sequence (C in Fig. 6, see Table S1 for its sequence) as the competitors. In the absence of competitors, both the un-induced (Lane 2 in Fig. 6) and flavone-induced (Lane 3 in Fig. 6) nuclear extracts formed two sequence-specific DNA-protein complexes (Band 1 and 2 in Fig. 6) with the probe. Both complexes were completely competed out by excess of cold probe (M, Lane 4 and 5 in Fig. 6), but not by the unrelated nonspecific sequence (C, Lane 6 in Fig. 6). The two DNA-protein complexes were 5.73- (Band 1) and 1.79-fold (Band 2) stronger in...
Lane 3 (which used the flavone-induced nuclear extracts for binding reaction) than in Lane 2 (which used the un-induced nuclear extracts for binding reaction) (Fig. 6). This suggests that flavone induction of *H. zea* fatbody cells significantly increases the nuclear concentrations of the yet to be characterized transcription factors that recognize and activate the XRE-Fla.

Among the three components of the XRE-Fla, the ARE-like element (M4) and the TAAT inverted repeat (M2) completely

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**Fig. 5.** Roles of XRE-Fla and its three components in the long and short CYP321A1-pGL3 constructs. The essential XRE-Fla (M, underlined) and its flanking sequence are showed in A, with its TAAT inverted repeat (M2), GCT mirror repeat (M3), and ARE-like element (M4) underlined. The key positions marking the fragments (M2, M3, M4, and M) to be internally deleted or mutated in the long and/or short constructs are indicated by arrowheads above the corresponding nucleotides. The sequences of the mutated M2, M3, and M4 in the three substitution mutation constructs (G–I) are also showed in A, with small case letters indicating altered nucleotides. For each set of internal deletion (B–F) and substitution mutation (G–I) constructs, a representative set of experiments, performed in triplicate, is showed in B–I, respectively. The basal relative firefly luciferase activities of the unchanged long or short construct were arbitrarily set to 1. Values sharing the same letter are not significantly different at *P* < 0.05 (Tukey’s HSD tests).
competed away band 2, whereas the GCT mirror repeat (M3) had no impact on band 2 formation (Lane 7—9 in Fig. 6). In terms of competing against band 1 formation, the XRE-Fla was the most efficient competitor (completely diminished it; Lane 5), followed by the ARE-like element (nearly diminished it; Lane 7), the TAAT inverted repeat (reduced its intensity by 37.5%), and the GCT mirror repeat (M3) (reduced its intensity by 26.5%). The competition pattern observed for band 1, but not band 2, is largely consistent with the impacts of internal deletion and/or mutation of the three XRE-Fla components on the basal and flavone-induced promoter activities and flavone inducibility of the long and short CYP321A1 promoter constructs (Fig. 5). This finding, together with the fact that band 1 was much more inducible than band 2 by flavone treatment (Lane 2—3 in Fig. 6), implies that the transcription factors that formed band 1 with the XRE-Fla are the major mediators for flavone regulation of CYP321A1 expression.

4. Discussion

When feeding on plants, insect herbivores continuously adjust the expression levels of their counterdefensive P450s to cope with escalating plant defenses by perceiving and transducing signals, particularly allelochemicals, from their host plants (Li et al., 2007; Li and Ni, 2009). As the initial step towards elucidation of the flavone regulatory cascade for induction of the allelochemical- and insecticide-metabolizing CYP321A1 (Sasabe et al., 2004; Niu et al., 2008; Wen et al., 2009; Rupasinghe et al., 2007), the promoter activities of varying length of CYP321A1 promoter progressive deletion, internal deletion and substitution mutation constructs were examined in a homologous H. zea fatbody cell line. These were used to map the cis-acting elements for the basal and flavone-induced expression of CYP321A1. The fact that the homologous H. zea fatbody cell line had the identical basal and flavone induced expression pattern of CYP321A1 with H. zea larvae (Fig. 2; Zeng et al., 2007; Niu et al., 2008; Wen et al., 2009) makes the cis elements characterized or located in this study ecologically relevant.

Progressive 3’ deletion analyses showed that the CYP321A1 5’ UTR but not its 5’-proximal coding sequence contains positive cis-acting elements required for its maximal basal and flavone-induced expression. This is consistent with the result of Brown et al. (2004), who found that CYP6B1 transcription was modulated by multiple elements in its 5’ UTR, and the result of van der Stoep et al. (2002), who confirmed that several cAMP-responsive elements (CREs) in the 5’ UTR of the class II trans-activator (CIITA) were involved in the activation of the CIITA promoter III. Since 5’UTRs are well known for their central roles in directing translational regulation of eukaryotic genes (Lawless et al., 2009), simultaneous analyses of the mRNA and activity levels of the luciferase reporter gene are necessary to clarify whether CYP321A1 5’ UTR enhances the transcription or/and translation of this gene. Two putative arthropod Iavr elements (CTAGTTG, +12 to +18, and CTACATC, +41 to +46), known to be stimulatory for the basal or induced transcriptions of some insect promoters (Smale, 2001; Smale and Kadonaga, 2003; Brown et al., 2004), are present within the CYP321A1 5’UTR (Fig. 3). Further deletion and/or substitution mutation analyses are needed to test if they are responsible for the dramatic enhancing effects on the basal activity conferred by the CYP321A1 5’ UTR sequence.

The statistically significant increases or reductions in the basal and flavone-induced promoter activities of two consecutive from the first two rounds of progressive 5’ deletions (Fig. 4C and D) strongly suggest that each of the following seven regions, including −79 to −119, −119 to −159, −159 to −199, −199 to −237, −278 to −310, −558 to −888, and −1218 to −1470, contains a major positive or negative cis-acting element. Specifically, the −79 to −119 region contains the essential element (i.e., XRE-Fla) required for both the basal and flavone-induced expression of CYP321A1 because deletion from −119 to −79 knocked out the basal and flavone-induced activities and flavone inducibility (Fig. 4D). Both the −159 to −199 and −278 to −310 regions contain an enhancer for its basal expression, whereas both the −119 to −159 and −558 to −888 regions contain an enhancer for its flavone-induced promoter activities. By contrast, the −199 to −237 region contains a silencer for its basal expression. The −1218 to −1470 region contains a silencer for both the basal and flavone-induced expression. In addition, there is a weak enhancer for flavone inducibility (increases not significant) in the −888 to −1218 region (Fig. 4C). Because both the 3’ sequence of the −1218 to −1470 region and the 5’ sequence of the −888 to −1218 region belong to HzIS1-3, it is possible that both the strong silencer and the weak enhancer are located in HzIS1-3. Such possibility is consistent with the overall weak silencing effect of HzIS1-3 revealed by internal deletion of HzIS1-3 (Fig. 4B).
specific DNA-protein complexes between the 36-bp fragment from −109 to −74 and the H. zea fatbody cell nuclear extracts (Fig. 6), demonstrate that the TAAT inverted repeat, GCT mirror repeat and ARE-like element constitute the essential XRE-Fla.

Prior to this study, two allelochemical response elements - xenobiotic response element to xanthotoxin (XRE-xan) and xenobiotic response element to aryl hydrocarbon receptor (XRE-AhR) - have been functionally characterized from both of the CYP6B1 and CYP6B4 promoters in heterologous Sf9 cells (Brown et al., 2004, 2005; Hung et al., 1996; McDonnell et al., 2004; Prapaipong, 1995; Petersen et al., 2003). The XRE-AhR found in the CYP6B1 promoter shares no significant sequence similarity with the XRE-Fla characterized in this study (Fig. 7). By contrast, the XRE-Xan from the CYP6B1 promoter shares the core nucleotides of ARE with the XRE-Fla (Fig. 7). Since both CYP6B1 and CYP321A1 can be induced by flavone and xanthotoxin (Berenbaum et al., 1990; Petersen et al., 2003; Sasabe et al., 2004; Zeng et al., 2007; Niu et al., 2008; Wen et al., 2009), it is logical to hypothesize that the two structurally different allelochemicals act as oxidants to induce the transcription of the two allelochemical-metabolizing P450 genes largely via their shared ARE. This hypothesis can be partially tested by determining if the XRE-xan is also required for flavone (and other allelochemicals) induction of CYP6B1 and if XRE-Fla is essential for induction of CYP321A1 by xanthotoxin and even other allelochemicals.

By examining the 36-bp essential XRE-Fla element, 5′-ATATA-TAATCGGGTCCAAATGATTCCAGAAAAA-3′, from both orientations, we identify another ARE-like element (i.e., 5′-ATATATATACTCC GTCCTAACAAAT-3′, called reverse ARE) that reads from 3′ to 5′ and consists 5′-part of the (bold and underlined nucleotides) of M4 (i.e., forward ARE), the GCT mirror repeat, and the TAAT inverted repeat. Therefore, the essential XRE-Fla element actually consists of two reversely oriented, overlapping (the bold and underlined nucleotides) ARE-like motifs. Because ARE is recognized and upregulated by increased nuclear accumulation of the nuclear factor-erythroid-2-related factor 2 (Nrf2) upon oxidative or electrophilic stress in vertebrates (Li, 2009; Suh et al., 2008; Trachootham et al., 2008), it is logical to hypothesize that induction of CYP321A1 by flavone and probably other allelochemicals is mediated by Cap’nCollar isoform C (CncC), the insect homolog of vertebrate Nrf2 (Kobayashi et al., 2002; Sykiotis and Bohmann, 2008). This hypothesis is supported by the EMSA data that showed stronger specific DNA-protein complex bands (suggesting higher nuclear concentrations of the corresponding transcription factors) in the nuclear extracts prepared from the flavone-induced H. zea fatbody cells (Lane 3 in Fig. 6) than in the nuclear extracts from un-induced H. zea fatbody cells (Lane 2 in Fig. 6). More experiments are needed to test this hypothesis and define the signaling cascades for allelochemical induction of CYP321A1 and other counterdefense genes in H. zea.

In summary, we have localized seven cis-acting elements in the CYP321A1 promoter regions and one in the CYP321A1 5′ UTR. The 36-bp essential element XRE-Fla characterized here represents to our knowledge the first flavone response element in insect herbivores. Previous studies confirm that evolutionary acquisition / retention of more counterdefense genes (Drosophila 12 Genomes Consortium, 2007; Li et al., 2003) and functional versatility of counterdefense genes (Li et al., 2004, 2007) allow polyphagous herbivores to cope with the diversity and unpredictability of plant defenses. Presence of at least eight cis regulatory elements in the 5′ UTR and promoter region of the H. zea P450 CYP321A1 leads us to propose that evolutionary acquisition / retention of more cis regulatory elements in the 5′UTR/promoter regions of counterdefense genes may contribute to polyphagy as well. Comparative localizations of cis elements in the 5′UTR/promoter regions of homologous pairs of generalist and specialist counterdefense genes are needed to test this hypothesis.

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Appendix. Supplementary material

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.ibmb.2010.09.003.

References


Fig. 7. Sequence comparison of the CYP321A1 XRE-Fla, the CYP6B1 XRE-AhR and the CYP6B1 XRE-xan. The CYP321A1 XRE-Fla is shown with its TAAT inverted repeat (−109 to −100), GCT mirror repeat (−98 to −93), and ARE-like element (−92 to −74) underlined and labeled as M2, M3, and M4, respectively. The core nucleotides of ARE shared by the CYP6B1 XRE-xan and the CYP321A1 XRE-Fla are shaded in grey. The CYP6B1 XRE-AhR shares no significant sequence similarity with the CYP321A1 XRE-Fla.


