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Correspondence and requests for materials should be addressed to X.L. (lxc@email.arizona.edu)

Common and unique *cis*-acting elements mediate xanthotoxin and flavone induction of the generalist P450 *CYP321A1*

Chunni Zhang[1,2], Andrew Wong[2], Yalin Zhang[1], Xinzhi Ni[3] & Xianchun Li[2]

[1]Key Laboratory of Plant Protection Resources and Pest Integrated Management, Ministry of Education, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China, [2]Department of Entomology and BIO5 Institute, University of Arizona, Tucson, AZ 85721, USA, [3]USDA-ARS Crop Genetics and Breeding Research Unit, Tifton, GA 31793, USA.

How polyphagous herbivores up-regulate their counterdefense genes in response to a broad range of structurally different allelochemicals remains largely unknown. To test whether this is accomplished by having more allelochemical-response elements or the similar number of functionally more diverse elements, we mapped out the *cis*-acting elements mediating the induction of the allelochemical-metabolizing CYP321A1 from the generalist *Helicoverpa zea* by xanthotoxin and flavone, two structurally distinct allelochemicals with very different encounter rate by this species. Seven xanthotoxin-responsive elements were localized by analyzing promoter activities of varying length of *CYP321A1* promoter in *H. zea* fatbody cells. Compared with the 5 flavone-responsive elements mapped out previously, there are four common elements (1 essential element, 2 enhancers, and 1 negative element) mediating induction of *CYP321A1* by both of the two allelochemicals. The remaining four elements (3 enhancers and 1 negative element), however, only regulate induction of *CYP321A1* by either of the two allelochemicals. Co-administration of the two allelochemicals resulted in an induction fold that is significantly lower than the expected additive value of the two allelochemicals. These results indicate that xanthotoxin- and flavone-induced expressions of *CYP321A1* are mediated mainly by the functionally more diverse common elements although the allelochemical-unique elements also play a role.

Evolution of host range has been a central topic in the field of plant–insect interactions[1–3]. Oligophagous insects, which are specialized on three or fewer families of host plants[4–6], typically encounter high levels of a narrow and predictable range of dietary allelochemicals[7]. Generalist herbivores, on the other hand, may encounter, by chance, any of a tremendous diversity of structurally distinct plant toxins, depending on which host plant they happen to live on. The diversity and uncertainty/unpredictability of plant defense allelochemicals constitute an incomparable toxic challenge for generalist herbivores.

Obviously, generalist herbivores must cope with the diversity and unpredictability of plant defenses to successfully exploit a wide range of host plants. One possible way to overcome the diversity of toxic plant allelochemicals idiosyncratically distributed among potential host plants is to have more counterdefense genes via gene duplication or fewer gene losses for detoxification of the diversity of plant allelochemicals[8]. That the two *Drosophila* specialists *D. sechellia* and *D. erecta,* which almost exclusively use decaying fruits of *Morinda citrifolia* and *Pandanus candelabrum*, respectively, have fewer allelochemical detoxification genes than the closely-related generalists *D. simulans*, and *D. yakuba*[9,10] support this hypothesis. This hypothesis is also supported by the finding of the allelochemical-metabolizing cytochrome P450 monooxygenase (P450) CYP6B genes from the generalists *Papilio glaucus*, *P. Canadensis* and *Helicoverpa zea* than from the specialists *P. polyxenes* and *Depressaria pastinacella*[7]. Another possible way to deal with the diversity of plant allelochemicals is that generalist counterdefense genes are functionally more diverse than their homologous counterparts in specialists. This notion is supported by the study of Li et al. (2004), who demonstrated that the generalist P450 CYP6B8 from *H. zea* is structurally more flexible and functionally more diverse than the specialist homolog CYP6B1 from *P. polyxenes*. Consistent with this notion, total glutathione-s-transferases (GSTs) isolated from the polyphagous *Spodoptera frugiperda* metabolize multiple organothiocyanates and

multiple α,β-unsaturated carbonyls, whereas the GSTs isolated from the specialist *Anticarsia gemmatalis* can metabolize only one organothiocyanate (benzyl thiocyanate)[11,12].

Having more counterdefense genes and more functions per counterdefense gene may provide generalists with some help to deal with the uncertainty and unpredictability of plant defenses. However, what really facilitates generalist to cope with the unpredictability challenge of plant defenses would be complex regulatory machinery that keep expressions of generalist counterdefense genes off or at a very low level, but dramatically upregulate a subset of these counterdefense genes when encountering certain allelochemicals. Consistent with this notion, allelochemical-metabolizing P450s in specialist herbivores are often constitutively expressed at a relatively high level, but can only be induced to certain extent by one or a very limited number of allelochemicals. Generalist P450s, on the other hand, are usually off or expressed at an extremely low level, but can be strongly induced by a range of allelochemicals. For example, transcripts of the specialist *P. polyxenes CYP6B1* are constitutively detectable and 2.9- to 3.7-fold inducible by the linear furanocoumarin xanthotoxin, the principal allelochemical present in the narrow host plants of this species[13], whereas transcripts of the generalist *P. glaucus CYP6B4* are barely detectable and 312-fold inducible by xanthotoxin[14]. In the more polyphagous *H. zea*, the homologous *CYP6B* genes are not only inducible by xanthotoxin, but also strongly induced by a broad range of structurally distinct allelochemicals including imperatorin (another furanocoumarin), indole-3-carbinol, chlorogenic acid (shikimate intermediate), flavone (flavone aglycone), jasmonic acid and salicylic acid (plant signaling molecules) and coumarin[15–18].

How a broad range of structurally different allelochemicals induces generalist counterdefense genes remains largely unknown. Two logical hypotheses may account for the inducibility of generalist counterdefense genes by multiple structurally different allelochemicals. First, generalist counterdefense genes may have more allelochemical-responsive elements in their promoter regions and each element mediates induction of counterdefense genes by a particular allelochemical. Alternatively, generalist counterdefense genes may have a similar number of allelochemical-responsive elements with specialist counterdefense genes, but each of the generalist elements is functionally more diverse and thus can mediate induction of counterdefense genes by multiple structurally different allelochemicals.

To examine the aforementioned two hypotheses, we chose to characterize and compare the *cis*-acting elements that mediate flavone and xanthotoxin induction of the generalist P450 *CYP321A1* in *H. zea*, a polyphagous lepidopteran of economic importance. Like CYP6B8 in *H. zea*, CYP321A1 metabolizes a wide range of allelochemicals and insecticides[19–21]. Transcripts of *CYP321A1* are constitutively low, but highly inducible by a broad range of plant allelochemicals including flavone and xanthotoxin[17–19,22]. Flavone and xanthotoxin are chosen because they are very different in terms of their chemical structure, distribution among plants, and ecological encounter rate by *H. zea*. The flavonoid flavone, a flavone aglycone, occurs in a wide range of host plant families of *H. zea*[23], and thus is frequently encountered by *H. zea*. In contrast, xanthotoxin, a linear furanocoumarin, is present in Apiaceae and Rutaceae, two occasional host plant families of *H. zea*[23]. We have recently identified/mapped out one essential element, three enhancers and one negative element that mediate flavone induction of *CYP321A1*[24]. In this study, we characterized/mapped out the *cis*-acting elements responsible for xanthotoxin induction of *CYP321A1* and compared their locations with those of the flavone-responsive elements identified in Zhang et al. (2010)[24]. We also compared the inducibility of *CYP321A1* by xanthotoxin, flavone, and mixture of xanthotoxin plus flavone. The data obtained support neither of the two hypotheses, but the integration of the two hypotheses.

Results

Characterization of the xanthotoxin-responsive elements in the *CYP321A1* promoter region. *Xanthotoxin inducibility of CYP321A1 in H. zea fatbody cells.* To confirm the xanthotoxin inducibility of *CYP321A1* in the homologous *H. zea* fatbody cell line BCIRL-HzFB33, we conducted RT-PCR gel analyses with mRNA samples extracted from the control and xanthotoxin-induced cells respectively. RT-PCR analyses shown that *CYP321A1* transcription in the *H. zea* fatbody cells was highly induced by xanthotoxin (Fig. 1). This indicates that the *H. zea* fatbody cells have the signaling machinery for using xanthotoxin as a signal to upregulate *CYP321A1* gene expression.

Roles of 5′UTR in xanthotoxin-induced expression of CYP321A1. To determine whether 5′UTR contains *cis*-acting elements important for xanthotoxin-induced expression of *CYP321A1*, a set of three *CYP321A1* promoter 3′ progressive deletion constructs, P(−1470/+84), P(−1470/+64) and P(−1470/−1) were transfected into *H. zea* fatbody cells and then induced with methanol (control) or 18.5 μM of xanthotoxin for 48 h. Compared to the promoterless *pGL3-basic* construct, all the three 3′ deletion constructs had significant basal and xanthotoxin-induced luciferase activities (Fig. 2A). The promoter plus 5′UTR construct P(−1470/+64) had the highest basal and xanthotoxin-induced promoter activity. Compared to that of P(−1470/+64), the promoter only construct P(−1470/−1) showed 79% decrease in the basal luciferase activity and 82% decrease in the xanthotoxin-induced luciferase activity, and the coding sequence-containing construct P(−1470/+84) showed 40% decrease in the basal luciferase activity and 42% decrease in the xanthotoxin-induced luciferase activity. The results suggest that 5′UTR sequence of *CYP321A1* is necessary for the maximal basal and xanthotoxin-induced expression of *CYP321A1*. The results also suggest that the 20-bp coding sequence (+65 to +84) have a negative effect on the basal and xanthotoxin-induced promoter activity.

Roles of HzIS1-3 in xanthotoxin-induced expression of CYP321A1. The wild type *CYP321A1* promoter sequence is 1470-bp long and contains a transposon named HzIS1-3 from −1348 to −1149[24,25]. Relative to the full-length promoter plus 5′UTR construct P(−1470/+64), deletion of *HzIS1-3* increased the basal promoter activity by

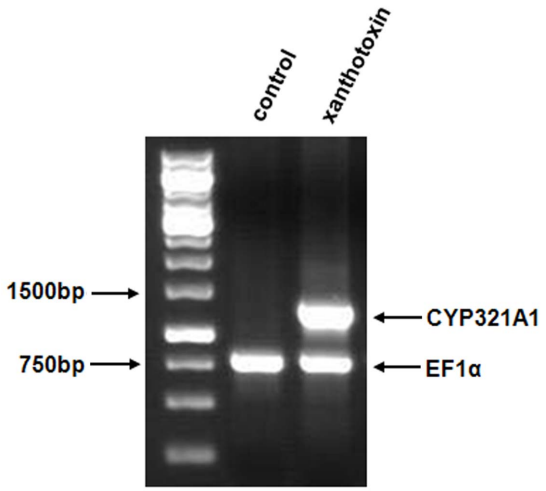

Figure 1 | *CYP321A1* expression in homologous *H. zea* fatbody cells. Homologous *H. zea* fatbody cells seeded onto a 6-well plate at 9×10^5cells/well were induced by 18.5 μM xanthotoxin or equal volume of methanol (control) for 48 h. Total RNA extracted from these cells were used as templates to quantify *CYP321A1* and elongation factor 1-α (EF-1α, reference gene) transcripts by RT-PCR gel analysis. Showed in this figure is a representative gel picture.

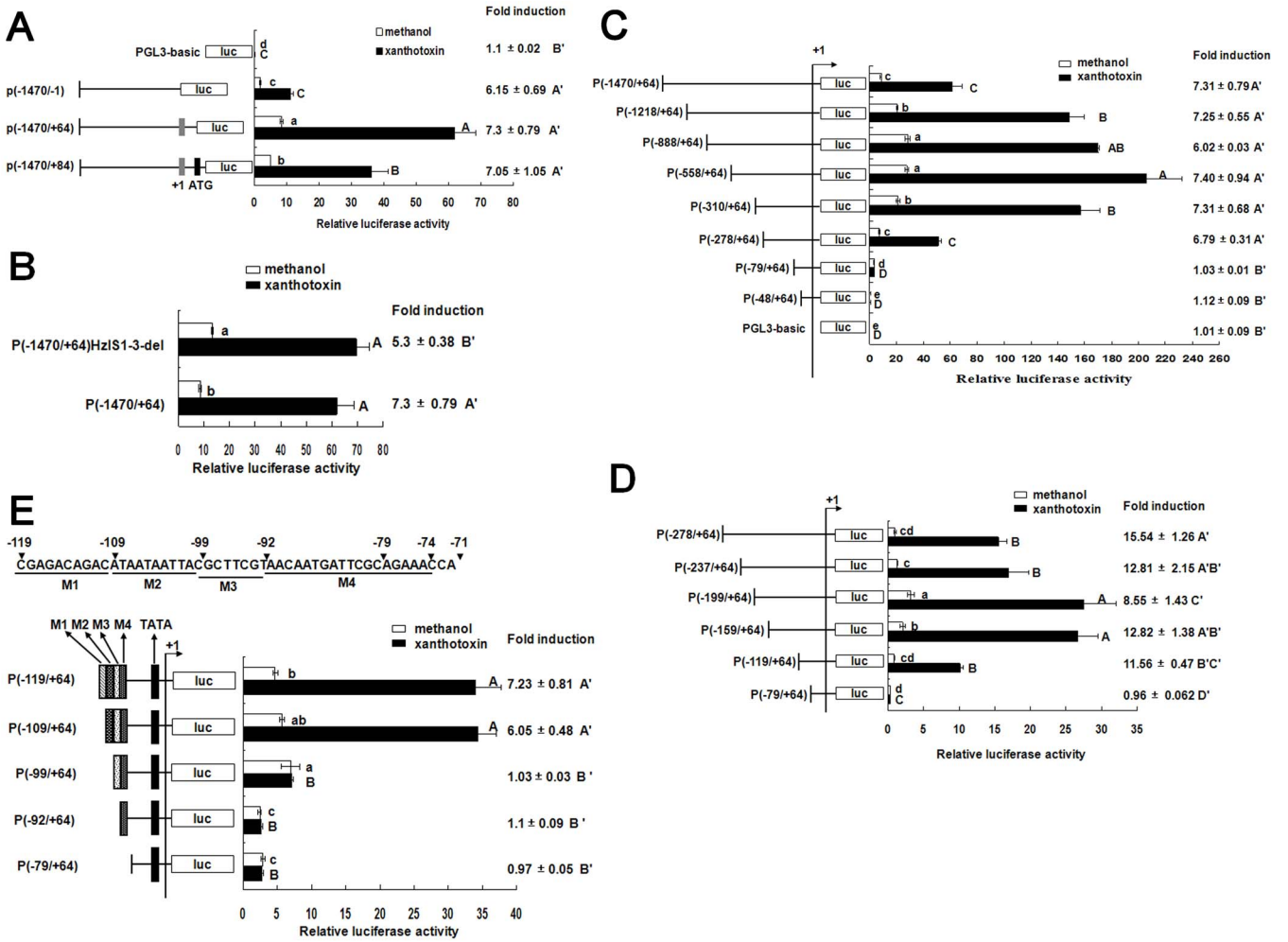

Figure 2 | Mapping of xenobiotic response element to xanthotoxin and other *cis* elements in *CYP321A1* promoter region by internal (B), progressive 3′ (A) and 5′ (C, D, E) deletions. The name of each *CYP321A1 promoter-pGL3* construct is composed of a "P" and a pair of parentheses that contain two numerals, separated by a slash, to specify the 5′ and 3′ positions of the corresponding promoter fragment [e.g., P(−1470/+64)]. For internal deletion or substitution mutation (see Fig. 3) constructs, the name of the deleted or mutated sequence and a "-del" or "-mut" are added after the parentheses [e.g., P(−1470/+64)HzIS1-3-del]. In each component of this composite figure, the names and schematic diagrams of the corresponding set of *CYP321A1* promoter-*pGL3* constructs (names only in B) are showed on the left side, with a white horizontal box representing the luciferase open reading frame and a grey vertical box (A) or a bent arrow plus +1 (C–E) representing the transcription start site +1. Homologous *H. zea* fatbody cells were co-transfected with the phRL-TK control plasmid and each *CYP321A1 promoter-pGL3* construct and then treated with 18.5 μM of xanthotoxin or equal volume of methanol as described in Materials and Methods. The basal (methanol-treated, white bars) and xanthotoxin-induced (black bars) relative firefly luciferase activities as well as the xanthotoxin induction folds of the corresponding set of *CYP321A1 promoter-pGL3* constructs are showed on the right side. The data and error bars represent the means and standard deviation of at least three independent transfections. Values sharing the same letter [small case letters for the basal activity, capital letters for the xanthotoxin-induced activity, and capital letters with a prime (e.g., A') for fold induction] are not significantly different at $P < 0.05$ (Tukey's HSD tests). A. Progressive 3′ deletions. B. The transposable element *HzIS1-3* deletion. C. The 1st round of progressive 5′ deletions D. The 2nd round of progressive 5′ deletions. E. The 3rd round of progressive 5′ deletions.

56.2% (significant) and the xanthotoxin-induced promoter activity by 12.4% (not significant; Fig. 2B). This data suggests that *HzIS1-3* insertion represses the basal but not the xanthotoxin-induced expression of *CYP321A1*.

Localization of cis-acting elements by progressive 5′ deletions. A series of eight *CYP321A1* promoter 5′ progressive deletion constructs were co-transfected into *H. zea* fatbody cells with phRL-TK control plasmid and treated for 48 h with methanol or 18.5 μM xanthotoxin. The deletion construct P(−558/+64) had the highest basal and xanthotoxin-induced promoter activities, which were significantly higher than those of the other 7 constructs except for P(−888/+64) (Fig. 2C). Progressive 5′ deletions from −1470 to −1218 (significant) and −888 to −558 (not significant) enhanced the basal and xanthotoxin-induced activities, from −1218 to −888 enhanced the basal activities (significant) and xanthotoxin-induced activities (not significant), whereas progressive 5′ deletions from −558 to −310 and −278 significantly reduced the basal and xanthotoxin-induced activities. Progressive 5′ deletion from −278 to −79 and −48 significantly reduced or abolished [P(−48/+64)] the basal activity and completely blocked xanthotoxin inducibility (Fig. 2C). These results indicate that there is one enhancer for the basal and xanthotoxin-induced expression of *CYP321A1* located between −278 to −310 and −310 to −558, respectively, and one negative element for the basal and xanthotoxin-induced expression of *CYP321A1* located between −1218 to −1470. But the essential *cis*-acting element necessary for the basal and xanthotoxin-induced expression of *CYP321A1* is present in the 200-bp region between −278 to −79, the same region that the essential flavone-responsive element XRE-Fla resides[24]. Because a xenobiotic response element to xanthotoxin

(XRE-Xan) was identified previously from the promoter regions of the *Papilio* P450s *CYP6B1* and *CYP6B4*[26–29] and that element (i.e. XRE-Xan) is not present in the 200-bp region localized above, we therefore refer to the essential element in the 200-bp region of the *CYP321A1* promoter hereafter as XRE-Xan1.

Fine mapping of XRE-Xan1. To more precisely map the essential XRE-Xan1, we compared the basal and xanthotoxin-induced promoter activities of a series of six progressive 5′ deletion constructs from −278 to −79 (Fig. 2D). Deletion from −278 to −237 only resulted in a slight increase in both the basal and xanthotoxin-induced promoter activities, whereas deletion from −237 to −199 significantly enhanced the basal and xanthotoxin-induced promoter activities. Deletion from −199 to −159 did not change the xanthotoxin-induced promoter activity but significantly decreased the basal promoter activity. Moreover, deletion from −159 to −119 significantly reduced the basal and xanthotoxin-induced activities, whereas deletion from −119 to −79 completely abolished the xanthotoxin-induced promoter activity (Fig. 2D). These data indicate that the region from −119 to −159 contains an enhancer for the basal and xanthotoxin-induced activity, whereas the region from −199 to −237 contains a negative element for the basal and xanthotoxin-induced activity. But the essential XRE-Xan1 is localized in the region from −119 to −79 or a few bp downstream of −79, exactly the same region as the essential XRE-Fla resides[24].

The region from −119 to −71 contains a direct repeat of "AGAC" motif from −116 to −109 (M1 in Fig. 2E), an inverted repeat of "TAAT" motif spaced by one nucleotide "A" from −109 to −100 (M2), a mirror repeat of "GCT" motif from −98 to −93 (M3), and the antioxidant-response element-like (ARE-like) element from −92 to −74 (M4). To determine which of the four repeats or motifs comprise the XRE-Xan1, we compared the basal and xanthotoxin-induced promoter activities of a series of five progressive 5′ deletion constructs from −119 to −79, each of them deletes one of the four repeats or motifs (Fig. 2E). Deletion from −119 to −109 had little effect on the basal and xanthotoxin-induced activities, whereas deletion from −109 to −99 completely blocked the xanthotoxin inducibility but did not significantly change the basal activity. Deletions from −99 to −92 and −79 further reduced the basal (significant) and xanthotoxin-induced (not significant) activities (Fig. 2E). These results suggest that the XRE-Xan1 completely overlaps with the XRE-Fla we recently defined[24], and is 36-bp long (from −109 to −74) and composed of the TAAT inverted repeat, the GCT mirror repeat and the ARE-like element.

To further address the roles of each component, we compared the basal and xanthotoxin-induced promoter activities of a set of internal deletion constructs, which delete any one of the three component or the whole XRE-Xan1 element from the short construct P(−278/+64) and/or the long construct P(−888/+64), with those of the constructs P(−278/+64) and/or P(−888/+64). Deletion of the TAAT inverted repeat (M2 in Fig. 3A) significantly decreased the xanthotoxin-induced promoter activity in both the short and long constructs and the basal promoter activity in the long construct but increased it in the short construct (Fig. 3B and C). Deletion of the GCT mirror repeat (M3) from the short construct reduced the basal activity by 65.1% and xanthotoxin-induced activity by 92.8%, causing negligible xanthotoxin inducibility (1.19 fold) (Fig. 3D). Deletion of the ARE-like element (M4) and the whole XRE-Xan1 element from both the short and long constructs not only abolished the xanthotoxin inducibility but also significantly reduced the basal promoter activity (Fig. 3E and F). These data indicate that the ARE-like element and the GCT mirror repeat are essential for both the basal and xanthotoxin-induced expression, whereas the TAAT inverted repeat is only necessary for the xanthotoxin inducibility but not for the basal expression.

Mutational analysis of the XRE-Xan1. To determine if mutations of the nucleotides and repeat structures of the three components affect the xanthotoxin inducibility in the same way as they impact the flavone inducibility[24], we compared the basal and xanthotoxin-induced promoter activities of the three multiple base pair substitution mutation constructs P(−109/+64)M2-mut, P(−888/+64)M3-mut and P(−888/+64)M4-mut with their wildtype counterparts (Fig. 3A, 3G, 3H and 3I). Mutation of the TAAT inverted repeat ATAATAATTA to AggggccTTA reduced the xanthotoxin-induced activity by 90.5%, but had no significant effect on the basal activity (Fig. 3G). Such effects were consistent with those of the corresponding internal deletion constructs (Fig. 3B and C). Mutation of the GCT mirror repeat CGCTTCG to CGaccaG, which alters its 4 central nucleotides but does not disrupt its mirror repeat structure, resulted in 45.7% reduction in the xanthotoxin-induced activity while increasing the basal activity by 119.7% (Fig. 3H). For comparison, internal deletion of this repeat resulted in significant reductions in both the basal and xanthotoxin-induced activities (Fig. 3D). These comparisons suggest that the 4 central nucleotides are probably less essential than the unmutated 5′ CG dinucleotide, the 3′ G and/or the mirror repeat, particularly for the basal activity. Consistent with internal deletion of the whole ARE-like element (Fig. 3E and F), mutation of the ARE-like element TAACAA-TGATTCGCAGAAA to TAACAAgaccTCttAGAAA resulted in the largest reductions in the basal and xanthotoxin induced activities and complete knockout of the xanthotoxin inducibility (Fig. 3I). This indicates that the altered nucleotides TGAT and GC are necessary for the basal and xanthotoxin-induced activities.

Comparison of xanthotoxin- and flavone-responsive elements. To determine if xanthotoxin and flavone use the same or different *cis*-acting elements to mediate expression of *CYP321A1*, we compared the locations of all the xanthotoxin-responsive elements mapped above and all the flavone-responsive elements identified in Zhang et al. (2010). As shown in Table 1, xanthotoxin uses five positive and two negative *cis* elements to mediate *CYP321A1* expression, where as flavone utilizes four positive and one negative elements to control *CYP321A1* expression. The two allelochemicals share the same essential element (−109 to −74, XRE-Xan1=XRE-Fla), two enhancers (+1 to +64 and −119 to −159) and one negative element (−1218 to −1470). Flavone has one unique enhancer localized from −558 to −888 that is not shared with xanthotoxin. Xanthotoxin, on the other hand, has 2 enhancers (−278 to −310 and −310 to −558) and 1 negative element (−199 to −237) that are not shared with flavone (Table 1).

Induction of *CYP321A1* by combination of xanthotoxin and flavone. To elucidate whether xanthotoxin and flavone regulate *CYP321A1* expression via common or different mechanisms, we analyzed the basal and allelochemical-induced promoter activities of *H. zea* fatbody cells transfected with the short construct P(−119/+64) or the full-length construct P(−1470/+64) in response to xanthotoxin (18.5 μM), flavone (18.5 μM), or their combination (18.5 μM + 18.5 μM). When cells were transfected with the short construct P(−119/+64), which contains only the essential element and the 5′ UTR enhancer shared by both allelochemicals (Table 1), xanthotoxin and flavone produced a 9.6- and 29.8-fold induction, respectively (Fig. 4A). The expected theoretical additive induction of the two allelochemicals would then be 39.4-fold (9.6+29.8). The combination of the two allelochemicals actually only elicited a 26.6-fold induction, which was similar to that of flavone but significantly lower than the theoretical additive value (Fig. 4A). When cells were transfected with the full-length construct P(−1470/+64) that contains both the common and xanthotoxin- or flavone-unique *cis*-acting elements (Table 1), xanthotoxin and flavone given alone generated a 7.3- and 17.9-fold induction, respectively (Fig. 4B). When the two

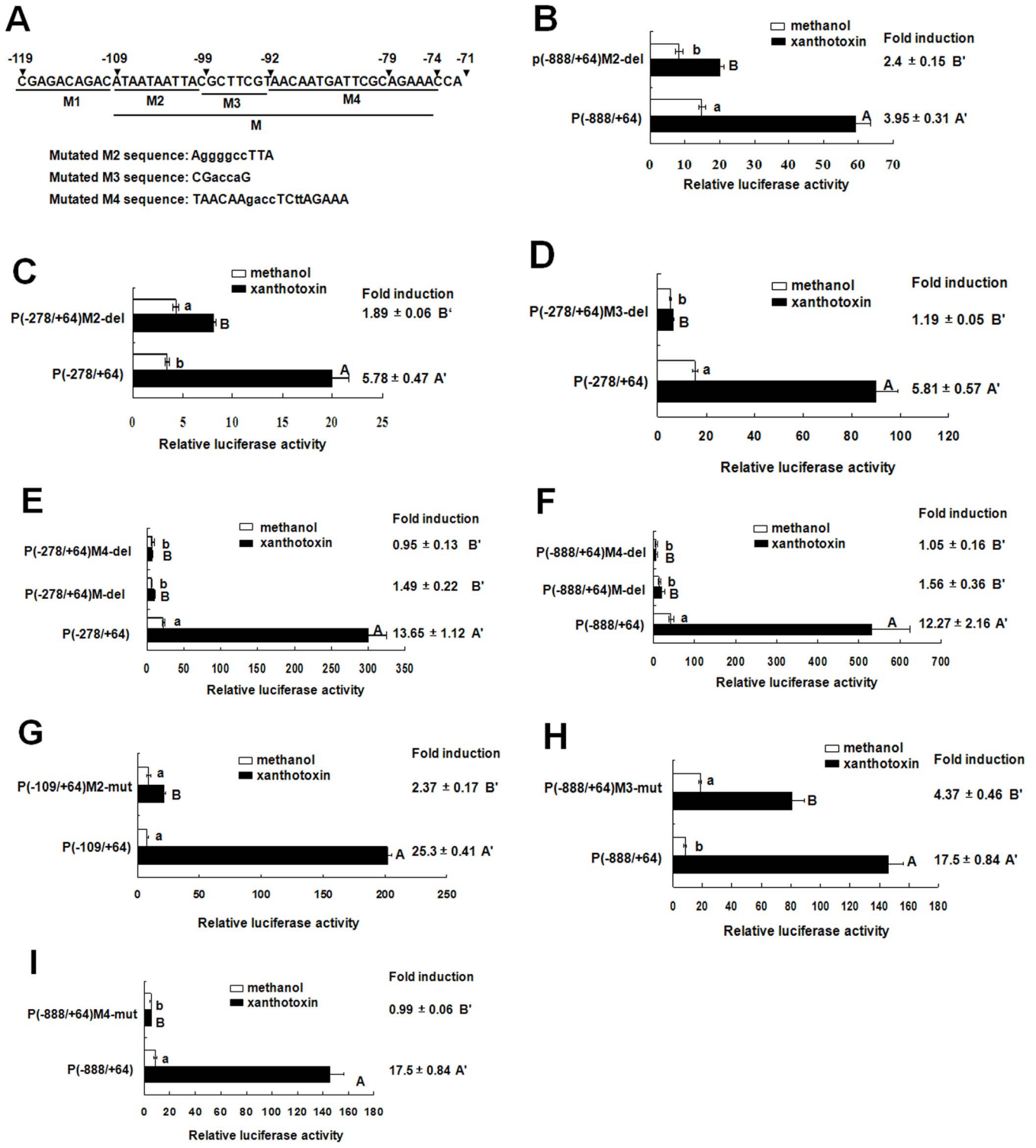

Figure 3 | Roles of xenobiotic response element to xanthotoxin and its three components in the long and short *CYP321A1-pGL3* constructs. The essential xenobiotic response element to xanthotoxin (M, underlined) and its flanking sequence are showed in A, with its TAAT inverted repeat (M2), GCT mirror 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. Values sharing the same letter are not significantly different at $P < 0.05$ (Tukey's HSD tests).

Table 1 | Xenobiotic-response elements (XREs) mediating flavone- and/or xanthotoxin-induced expression of *CYP321A1*

XRE-containing regions	Roles in flavone induction*	Roles in xanthotoxin induction
1 to 64 (=5′ UTR)	Enhancer	Enhancer
−74 to −109	Essential element, XRE-Fla	Essential element, XRE-Xan1
−119 to −159	Enhancer	Enhancer
−199 to −237	No roles	Negative element
−278 to −310	No roles	Enhancer
−310 to −558	No roles	Enhancer
−558 to −888	Enhancer	No roles
−1218 to −1470	Negative element	Negative element

*Mapping of these elements has been published in Zhang et al. (2010).

allelochemicals were given concomitantly, a 13.7-fold induction was observed. This value was significantly less than the expected theoretical additive induction of the two allelochemicals (7.3 + 17.9 = 25.2 fold) and that of flavone (P=0.038<0.05) (Fig. 4B).

Discussion

Relative to specialist herbivores, generalist herbivores face a tremendous diversity and unpredictability of toxic plant allelochemicals[7]. The capacity to induce their counterdefense genes in response to a wide range of structurally distinct allelochemicals is essential for generalist herbivores to overcome the uncertainty and unpredictability of plant defenses. This study was conducted to test whether this capacity is achieved by having more allelochemical-response elements ("more element" hypothesis) or similar number of functionally more diverse allelochemical-response elements ("more function" hypothesis).

By analyzing/comparing the basal and xanthotoxin-induced promoter activities of various *CYP321A1* promoter deletion and mutation constructs (Fig. 2 and 3), we localized a set of seven xanthotoxin-responsive *cis*-acting elements, including one essential element, four enhancers and two negative elements (Table 1). Compared with the flavone-responsive elements identified in Zhang et al. (2010)[24], four out of the seven xanthotoxin-responsive elements, including the essential XRE-Xan1 (i.e. XRE-Fla, −74 to −109), the two proximal enhancers (5′UTR and −119 to 159) flanking the essential element, and the distal negative element (−1218 to −1470), mediate induction of *CYP321A1* by both xanthotoxin and flavone (Table 1), two structurally distinct plant allelochemicals with very different ecological encounter rates by *H. zea*. But the remaining three elements, including two enhancers (−278 to −310 and −310 to −558) and one negative element (−199 to −237), only regulate xanthotoxin induction of *CYP321A1* and play no roles in flavone induction of *CYP321A1*. Moreover, there is a flavone-response enhancer (−558 to −888) that is not involved in xanthotoxin induction of *CYP321A1* (Table 1). These data support neither the "more elements" hypothesis nor the "more function" hypothesis. Instead, the data support the integration/combination of the two hypotheses. This is because half of the eight allelochemical-response elements listed in Table 1 can mediate induction of *CYP321A1* by the two different allelochemicals, whereas the other half can only regulate xanthotoxin or flavone induction of *CYP321A1*.

Among the eight allelochemical-responsive elements mediating flavone and/or xanthotoxin induction of *CYP321A1*, only the essential element was precisely mapped and characterized. The fact that internal deletion and mutation of the three components of the essential element resulted in the similar effects on the xanthotoxin-induced (Fig. 3) and flavone-induced[24] promoter activities suggests that the essential element mediates flavone and xanthotoxin induction of *CYP321A1* via the same trans-regulatory mechanism or transcription factor(s). If such is the case, simultaneous administration of the two allelochemicals to *H. zea* fatbody cells transfected with the short construct P(−119/+64) containing only the essential element

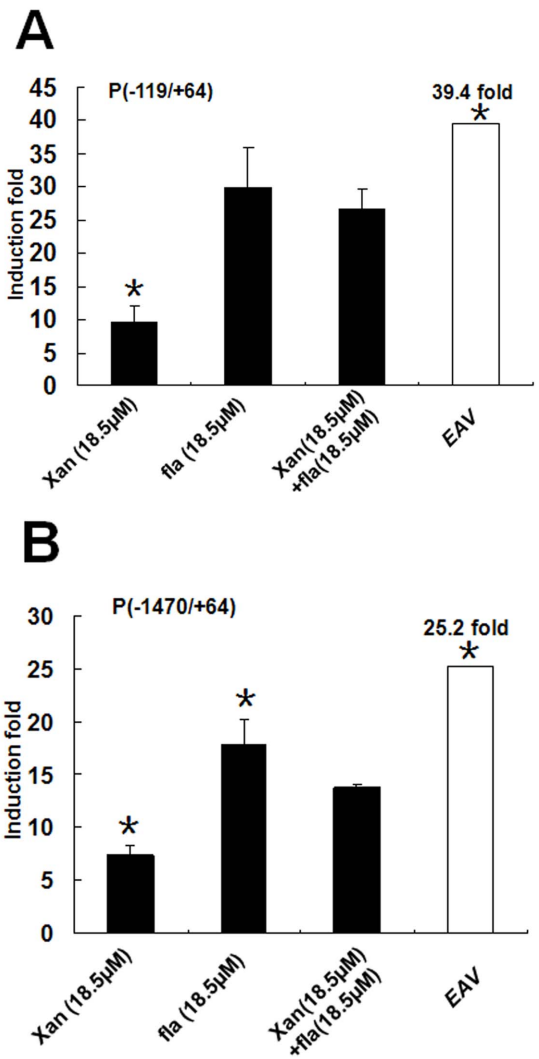

Figure 4 | Induction of *CYP321A1* by combination of xanthotoxin and flavone. *CYP321A1 promoter-pGL3* constructs p(−119/+64) (A) and p(−1470/+64) (B) were transfected into the *H. zea* fatbody cells, Sixteen hours post-transfection, the cells were treated with the methanol control, 18.5 μM flavone, 18.5 μM xanthotoxin or 18.5 μM flavone with 18.5 μM xanthotoxin. After 48 h, luciferase activities were measured using a dual luciferase assay system. The data and error bars represent the mean induction folds and standard deviation of at least three independent transfections. Asterisks represent significant difference between the two allelochemicals given concomitantly and the two allelochemcials given alone or the expected additive value (EAV) of the two allelochemicals at $P < 0.05$ (Tukey's HSD tests).

and the shared 5′ UTR enhancer would compete for the uncharacterized common transcription factors and inhibit each other's induction effects. As a result, combination of the two allelochemicals would produce an induction fold that is very near the induction fold of the better inducer flavone, but significantly smaller than the expected theoretical additive value of the two inducers using two different and independent regulatory mechanisms. The fact that this is exactly what we observed by co-administration of xanthotoxin and flavone to cells transfected with the short construct P(−119/+64) (Fig. 4A) confirms that the same trans-regulatory mechanism mediates xanthotoxoin and flavone induction of *CYP321A1* via the essential element.

When the full-length construct P(−1470/+64) was used to transfect *H. zea* fatbody cells, the induction fold elicited by combination of the two allelochemcials was not only significantly lower than the expected theoretical additive value of the two inducers, but also significantly lower than that of the superior inducer flavone (Fig. 4B). Given that the full-length construct P(−1470/+64) contains both the four shared elements and the four xanthotoxin- or flavone-unique elements, this result indicates that the two structurally different allelochemicals induce *CYP321A1* expression mainly via their four shared elements, especially the essential element. Because the essential element is composed of two reversely orientated, overlapping ARE-like motifs[24] and ARE is activated by nuclear translocation of the nuclear factor-erythroid-2-related factor 2 (Nrf2) upon oxidative or electrophilic stimulus in vertebrates[30–32], we hypothesize that the two allelochemicals regulate *CYP321A1* expression largely by generating oxidative or electrophilic stress. Further experiments are needed to test this hypothesis and to elucidate how oxidative or electrophilic stress is generated from the two allelochemicals.

Methods

Total RNA preparation and RT-PCR. The homologous *H. zea* fatbody cell line BCIRL-HzFB33, generously provided by Dr. Cynthia L. Goodman (BCIRL, USDA, ARS), was routinely maintained with Ex-cell 420 insect serum-free medium (SAFC Bioscience, 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[24]. To confirm the xanthotoxin inducibility of *CYP321A1* in this cell line, cells seeded onto a 6-well plate (9×10^5cells/well) containing 2 ml supplemented medium were treated with methanol (control) or xanthotoxin (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)[29] and our preliminary experiments (data not shown). Total RNA was isolated from these cells using TRIzol® LS Reagent (Invitrogen) according to manufacture's instructions. The RT-PCR was performed as described in Zhang et al. (2010)[24].

Construction of *CYP321A1* promoter-pGL3 constructs. 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, were made by PCR amplifications as described in Zhang et al. (2010).

Transient transfection and dual luciferase assay. *H. zea* fatbody cells seeded onto a 12-well plate (9×10^5cells/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 (Invitrogen; 8 μl per well). Sixteen hours post-transfection, xanthotoxin (at a final concentration of 18.5 μM), flavone (18.5 μM), xanthotoxin (18.5 μM) plus flavone (18.5 μM) 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 deviation of three or four independent transfections of one representative experiment. The induction folds reported are expressed as a ratio of the normalized xanthotoxin-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.

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Author contributions

X.C.L. and C.N.Z. conceived and designed the experiments. C.N.Z. and A.W. performed the experiments. C.N.Z. and X.C.L. analyzed the data. X.C.L., C.N.Z., Y.L.Z. and X.Z.N. wrote the paper.

Additional information

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