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Jasmonate and salicylate induce expression of herbivore cytochrome P450 genes

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Jasmonate and salicylate are plant-produced signals that activate plant defence genes after herbivory[1–3] or pathogen[4] attack. Amplification of these signals, evoked by either enemy attack or experimental manipulation, leads to an increase in the synthesis of toxic compounds (allelochemicals)[5–8] and defence proteins[6,9,10] in the plants. Although the jasmonate and salicylate signal cascades activate different sets of plant defence genes[10], or even act antagonistically[11,12], there is substantial communication between the pathways[2,3,13]. Jasmonate and salicylate also contribute to protecting plants against herbivores by causing plants that experience insect damage to increase their production of volatile molecules that attract natural enemies of herbivorous insects[14]. In response to plant defences, herbivores increase their production of enzymes that detoxify allelochemicals, including cytochrome P450s (refs 15, 16). But herbivores are potentially vulnerable to toxic allelochemicals in the duration between ingesting toxins and induction of detoxification systems. Here we show that the corn earworm *Helicoverpa zea* uses jasmonate and salicylate to activate four of its cytochrome P450 genes that are associated with detoxification either before or concomitantly with the biosynthesis of allelochemicals. This ability to 'eavesdrop' on plant defence signals protects *H. zea* against toxins produced by host plants.

The corn earworm, *H. zea*, is broadly polyphagous, with over 100 known host plants including herbs, shrubs and other low-lying vegetation. We chose *H. zea* to address the general issue of whether herbivorous insects can activate their enzymes that metabolize

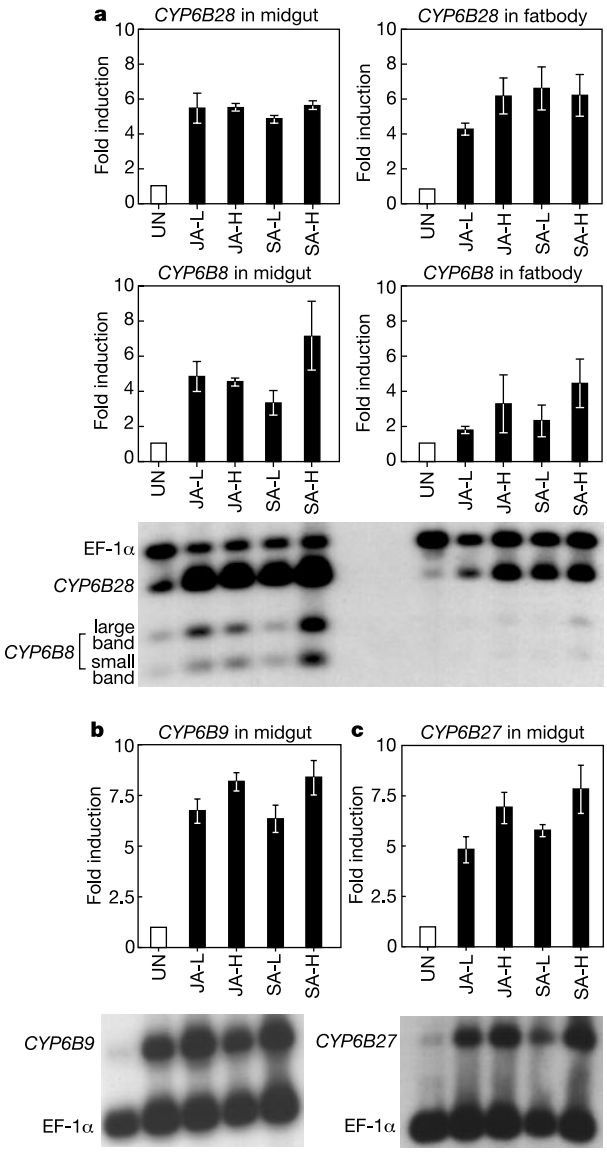

Figure 1 *CYP6B* gene expression in *H. zea* in response to jasmonate (JA) and salicylate (SA). **a**, *CYP6B8/CYP6B28*; **b**, *CYP6B9*; **c**, *CYP6B27*. Total midgut or fatbody RNAs from five different diet treatments (UN, control diet; JA-L, 2.9 $\mu g\ g^{-1}$ JA; JA-H, 290 $\mu g\ g^{-1}$ JA; SA-L, 12 $\mu g\ g^{-1}$ SA; SA-H, 1.2 $mg\ g^{-1}$ SA) were separately amplified by RT–PCR and analysed as described in Methods. The average fold induction and standard deviations (error bars) for three independent RT–PCR amplifications are shown in the histograms; an autoradiogram of a representative blot is shown below.

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Table 1 **Effects of jasmonate and salicylate exposure on growth rate, weight gain and mortality of *H. zea***

Treatments	Duration of fourth instar (d)	Duration of fifth instar (d)	Mortality on day 3 (%)	Weight gain on day 3 (mg)	Pupal weight (mg)	Final mortality (%)	Final pupation rate (%)
No exposure							
Control diets	1.5 ± 0.5 d	5.5 ± 0.5 e	0.0 ± 0.0 b	384.4 ± 25.6 a	346.4 ± 13.6 a	15.6 ± 5.1 d	84.3 ± 15.1 abc
Xanthotoxin diet	3.8 ± 0.6 b	11.7 ± 0.3 b	8.9 ± 1.9 b	102.2 ± 28.3 bc	225.3 ± 16.5 b	30.0 ± 10.0 bcd	70.0 ± 10.0 cd
Celery leaves	5.7 ± 0.3 a	14.7 ± 0.6 a	33.3 ± 15.3 a	47.6 ± 2.7 d	183.2 ± 23.4 c	86.7 ± 11.6 a	12.8 ± 6.3 e
JA-L							
Control diets	1.5 ± 0.5 d	5.5 ± 0.5 e	0.0 ± 0.0 b	396.1 ± 7.8 a	348.2 ± 24.3 a	6.7 ± 5.7 d	93.3 ± 5.8 a
Xanthotoxin diet	2.5 ± 0.5 c	9.5 ± 0.5 c	0.0 ± 0.0 b	78.9 ± 15.4 bcd	226.6 ± 2.5 b	26.7 ± 11.6 bcd	73.3 ± 11.6 bcd
Celery leaves	2.5 ± 0.5 c	8.5 ± 0.5 d	3.0 ± 5.3 b	84.5 ± 17.9 bcd	178.1 ± 39.5 c	48.3 ± 16.1 bc	48.7 ± 20.1 d
SA-L							
Control diets	1.5 ± 0.5 d	4.7 ± 0.6 e	3.3 ± 5.8 b	382.1 ± 50.3 a	372.9 ± 11.6 a	10.0 ± 10.0 d	89.9 ± 10.0 ab
Xanthotoxin diet	2.5 ± 0.5 c	9.7 ± 0.6 c	0.0 ± 0.0 b	113.1 ± 9.3 b	232.7 ± 6.5 b	23.3 ± 5.8 cd	76.7 ± 5.8 bcd
Celery leaves	2.5 ± 0.5 c	10.8 ± 0.3 b	7.0 ± 6.1 b	69.9 ± 15.5 cd	166.1 ± 15.6 c	49.9 ± 10.0 b	50.0 ± 10.0 d

Effects were measured from fourth instar to pupae on celery leaves and on control and 0.5% xanthotoxin diets. The diets contained 2.9 $\mu g\,g^{-1}$ JA (JA-L) or 12 $\mu g\,g^{-1}$ SA (SA-L). Data as percentages were arcsine transformed before analysis. Results are the untransformed means ± s.d. In each column, means followed by different letters are significantly different ($P < 0.05$, modified LSD *t*-test).

allelochemicals in response to the plant signal molecules jasmonate and salicylate before the accumulation of plant defence compounds. Transcripts of four *H. zea* cytochrome P450 (P450) genes are inducible by furanocoumarins, chlorogenic acid, indole-3-carbinol and flavone[16,17], which suggests that these genes are involved in detoxifying a range of plant allelochemicals. But these four P450 genes are not universally inducible by all allelochemicals of host plants; for example, gossypol, quercetin and rutin do not induce their transcription[17]. Baculovirus-mediated expression of one of these proteins, CYP6B8, has shown that this protein can metabolize xanthotoxin (221.1 pmol per ml of baculovirus-expressing cell culture per min; unpublished data)—a furanocoumarin that is present in many host plants of *H. zea* and whose biosynthesis is stimulated by jasmonate and methyl jasmonate[7].

In *Apium graveolens* (celery)[7,18], a host plant of *H. zea*[19], xanthotoxin and the related furanocoumarin bergapten begin to accumulate after 24 h and reach maximal concentrations (representing a 40–70-fold increase) 4–6 d after the application of jasmonate and methyl jasmonate[18]. Accordingly, we fed fifth instars of *H. zea* for 48 h with either artificial diets supplemented with jasmonate and salicylate (at two concentrations for each chemical) or control diets, and then examined expression of four P450 genes, *CYP6B8* (AF102263), *CYP6B9* (AF140278), *CYP6B27* (AF285829) and *CYP6B28* (AF285186), in the midgut and fatbody—the principal sites of allelochemical detoxification in this species[17].

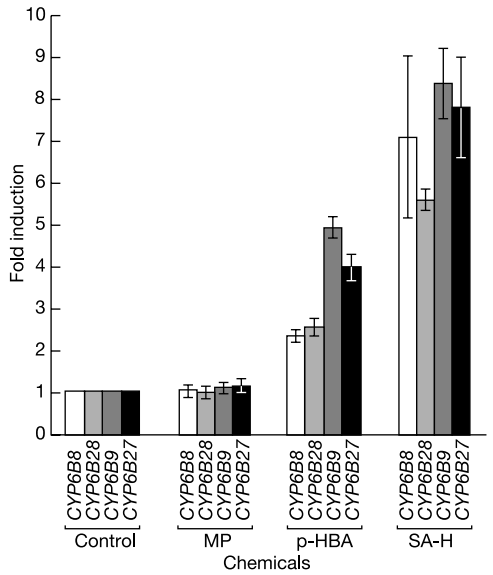

Figure 2 *CYP6B* expression in response to salicylate (SA) and SA-related *p*-hydroxybenzoic acid and methylparaben. Total RNAs from caterpillars fed on diets containing 1.2 mg g^{-1} SA (SA-H), 1.2 mg g^{-1} methylparaben (MP) or 1.2 mg g^{-1} *p*-hydroxybenzoic acid (p-HBA) were amplified by RT–PCR and analysed as described in Methods. The relative induction and standard deviations for three independent RT–PCR amplifications are shown.

Among the P450 transcripts examined, *CYP6B8* and *CYP6B28* (99% amino acid identity), a pair of highly conserved paralogues[20], were simultaneously amplified by polymerase chain reaction with reverse transcription (RT–PCR), differentiated by digestion with *Xmn*I, and quantified by gel blot analysis (Fig. 1a). In midguts, *CYP6B28* transcripts were induced about 5.0-fold by jasmonate and salicylate irrespective of the concentration, whereas *CYP6B8* transcripts were induced about 4.5-fold by either concentration of jasmonate, 3.3-fold by the low concentration of salicylate and 7.1-fold by the high concentration of salicylate (Fig. 1a). In fatbody, *CYP6B28* transcripts were induced about 6.0-fold by either concentration of salicylate, 4.2-fold by the low concentration of jasmonate, and 6.2-fold by the high concentration of jasmonate; transcripts of *CYP6B8* were increased to a lesser extent by jasmonate and salicylate (Fig. 1a).

The more divergent *CYP6B9* and *CYP6B27* transcripts derived from another pair of paralogous P450 genes (87% amino acid identity with *CYP6B8*), whose expression is restricted to midguts[17], were separately detected by RT–PCR gel blot analysis (Fig. 1b, c). In midguts, *CYP6B9* transcripts were induced 6.0-fold by low concentrations and 8.0-fold by high concentrations of jasmonate and salicylate (Fig. 1b), whereas *CYP6B27* transcripts were induced 4.8-fold and 5.8-fold by low concentrations of jasmonate and salicylate, respectively, and 6.9-fold and 7.8-fold by high concentrations of jasmonate and salicylate, respectively (Fig. 1c). These results show clearly that expression of *CYP6B* is activated in the midgut and fatbody of *H. zea* at the low concentrations of jasmonate and salicylate that are associated with pest damage and allelochemical induction in its host plants[4,21].

To assess the specificity of this induction response, we tested further the induction of these P450 genes in response to two salicylate-related chemicals at equivalent concentrations to high concentration of salicylate. Methylparaben, which differs from salicylate in the position of its hydroxy group and in having an additional methyl ester group, did not induce any of the *CYP6B* genes examined. Not surprisingly, *p*-hydroxybenzoic acid, which differs from salicylate only in the position of its hydroxy group, acted as a weaker inducer than salicylate and increased the amounts of *CYP6B8* and *CYP6B28* transcripts roughly 2.0-fold, and the amounts of *CYP6B9* and *CYP6B27* transcripts 4.0–5.0-fold (Fig. 2). These results indicate that the degree of activation of *H. zea CYP6B* genes by salicylate, jasmonate and related compounds is dependent on structural features of these signal molecules.

To test whether an increase in endogenous amounts of signal substances in plants that occurs before allelochemical biosynthesis is sufficient to induce transcriptional expression, we allowed starved fourth instars of *H. zea* to damage celery leaves and then determined the ability of these leaves to activate transcription of *CYP6B* in a

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second set of fifth instars 2 and 4 h after damage. Compared with leaves from two undamaged control plants, which did not induce *CYP6B* expression, leaves from all of the plants that had been attacked for 2 and 4 h induced expression of *CYP6B28*, *CYP6B9* and *CY6B27* (Fig. 3). Analysis of these plants indicated that, in a background of up to twofold constitutive differences in furanocoumarin content and composition among the test plants, no induced accumulation of furanocoumarin occurred. The differences in *CYP6B* expression were not correlated with variations in furanocoumarin between plants (Fig. 3), which indicated that the activation of *CYP6B* transcription resulted from an induction of jasmonate and/or other signal substances caused by the feeding behaviours of the first set of larvae. These data provide direct evidence that *H. zea* can intercept the plant defence signals elicited by its own feeding activity.

To determine whether activation of P450 genes in advance of exposure to furanocoumarins confers protection on *H. zea*, we compared the survival and growth of fourth instars that had prior exposure to low concentrations of jasmonate and salicylate for 12 h on celery leaves, 0.5% xanthotoxin diets, or control diets against that of control larvae that had not been exposed in advance to signal substances. Two-way analysis of variance (ANOVA) and multiple comparison tests on mortality, weight gain, growth rate and pupation success (Table 1) indicated that caterpillars exposed to jasmonate and salicylate survived better on celery leaves and 0.5% xanthotoxin diets for all parameters. On control diets, however, there were no significant differences in all parameters among the three treatments. These results suggest that the 'signal-eavesdropping' capability provides *H. zea* with prophylactic protection against plant defences at no additional cost to fitness in the absence of plant defences.

Reciprocal phenotypic responses characterize many antagonistic ecological interactions; if such reciprocal phenotypic change results from adaptive plasticity in the interacting species, then coevolutionary interactions may result in the evolution not only of fixed adaptations but also of phenotypic plasticity[22]. The induction of P450 counterdefence genes in herbivores in response to plant signal substances that are themselves inducible by herbivore damage might be an example of such phenotypic plasticity. Although it is well known that herbivorous insects can enhance the expression of detoxification enzymes (counterdefences) in the presence of plant allelochemicals (plant defences)[15–17,23,24], we have shown here that *H. zea* responses to plant damage are more sophisticated than was thought previously. By responding to plant signal molecules as well as the end-product allelochemicals, insects have the capacity to equip themselves before (or concomitant with) the accumulation of toxic concentrations of plant defence compounds. Although several examples have been found of plants using insect-derived signal substances to regulate their defence pathways[1–3], this represents to our knowledge the first example of the use by insects of plant signal molecules to regulate their defence systems against plant allelochemicals.

The ability to use plant signal molecules as cues for activating a detoxification system may be of particular value to a broadly polyphagous herbivore such as *H. zea*. In contrast to oligophagous species, which encounter a relatively narrow and generally predictable range of plant allelochemicals, generalized herbivores may encounter any of several biosynthetically distinct compounds depending on host plant choice[25]. Few commonalities exist among the biosynthetic pathways that generate these plant defence compounds other than the fact that they share jasmonate or salicylate as initiating signals. The ability of a generalist to respond

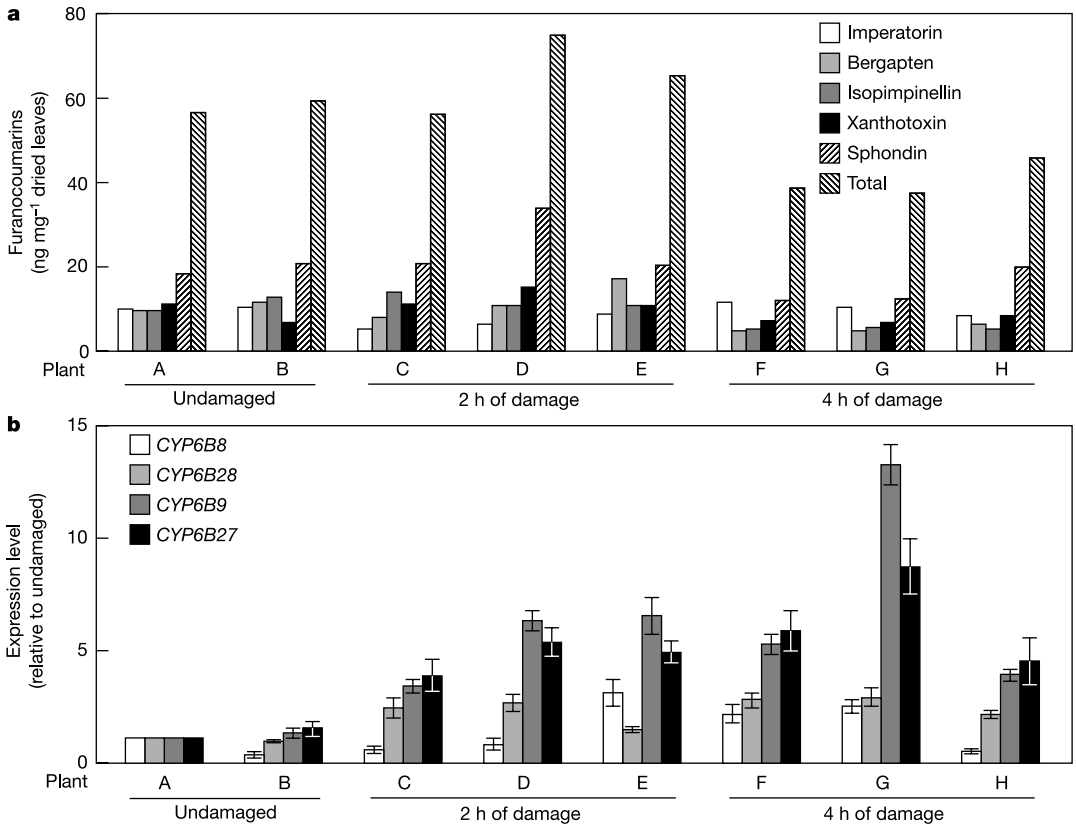

Figure 3 *CYP6B* activation by feeding on damaged celery leaves. **a**, Content of total and individual furanocoumarins for each plant. **b**, Relative induction and standard deviations for three independent RT–PCR amplifications. Total RNAs from fifth instars fed for 48 h on leaves that were previously undamaged or damaged for 2 or 4 h by starved fourth instars were amplified by RT–PCR and analysed as described in Methods.

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to these signals by upregulating several detoxification genes may maximize its ability to counter its host's response to damage, irrespective of taxon. □

Methods

Test insects

An insecticide-susceptible laboratory strain of *H. zea*, provided by B. R. Barrido (Abbott Laboratories), was used in all studies. We kept insects in an insectary maintained at 28 °C in a 16:8 h light:dark cycle on a semisynthetic control diet containing wheatgerm[26].

Signal chemical induction treatment

Artificial diets containing 2.9 or 290 $\mu g\,g^{-1}$ jasmonate (Sigma), 12 $\mu g\,g^{-1}$ or 1.2 $mg\,g^{-1}$ salicylate (99%, Aldrich), 1.2 $mg\,g^{-1}$ methylparaben (Sigma), or 1.2 $mg\,g^{-1}$ *p*-hydroxybenzoic acid (Sigma) were provided to 30 newly moulted fifth instars. The low concentrations of jasmonate and salicylate were selected on the basis of endogenous amounts of jasmonate and salicylate found in the host plants of *H. zea*[4,21] and the high concentrations were selected to maximize the likelihood of detecting an upper limit on the response. After 48 h, midguts and fatbodies were dissected out and total RNAs were isolated from each type of tissue using guanidine-HCl extraction[27] and then resuspended in diethyl pyrocarbonate (DEPC)-treated water.

Celery damage and induction treatment

We grew nine celery plants individually in pots under laboratory conditions for 3 weeks to ensure that they were free of herbivore and pathogen infestation. Eight of them were free of infestation and were assigned randomly to one of three groups: undamaged control, 2 h of damage and 4 h of damage. For each plant, four stems with fully expanded pairs of leaflets and a terminal leaflet were chosen for treatment. On each stem, two fourth instars that had been starved for 4 h were confined to the second pair of leaflets by two small clip cages, with one larva per leaflet. For the undamaged controls, clip cages without larvae were placed on the second pair of leaflets on each stem for 4 h. After damage treatments, the second pair of leaflets was removed from each treated stem. We used one damaged leaflet to feed a newly moulted fifth instar that had been starved for 4 h. We pooled another leaflet with the other three leaflets from the same plant, oven-dried them at 50 °C for 24 h and used them for furanocoumarin determination. After 48 h of feeding on the damaged leaves, larvae were killed and the midguts and fatbodies were removed. The midguts from the four larvae fed the damaged leaves from the same plant were pooled together and total RNA was isolated as described[27].

RNA and furanocoumarin analysis

We carried out RNA isolation and RT–PCR gel blot analyses as described[17]. For each RNA sample, three independent RT–PCR amplifications were carried out. For furanocoumarin assay, all leaf samples were weighed separately and ground to a fine powder with a plastic rod inside 1.5-ml Eppendorf tubes. Furanocoumarins were extracted, separated and quantified as described[28].

Jasmonate and salicylate protection bioassay

Newly moulted fourth instars (270) from the University of Illinois laboratory colony were divided randomly into three groups (90 larvae per group) and reared individually in plastic cups with fresh control diets or supplemented diets containing 2.9 $\mu g\,g^{-1}$ jasmonate or 12 $\mu g\,g^{-1}$ salicylate. After 12 h of exposure to plant signal molecules, each group was divided further into three subgroups (30 larvae per subgroup, three replicates of 10 insects) that were transferred to plastic cups with fresh control diets, diets containing 0.5% xanthotoxin, or celery leaves. Initial weights were recorded for every individual. All larvae were weighed again on the third day after transfer to experimental diets to determine weight gain. We monitored survival and developmental stage daily until all larvae had either pupated or died. Differences in weight gain, duration of fourth and fifth instars, mortality, pupal weight and percentage of pupation among the treatments were evaluated by ANOVA, followed by modified least significant difference test (LSD *t*-test), with the significance level set at $P < 0.05$ using the SAS statistics program.

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A biological role for prokaryotic ClC chloride channels

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An unexpected finding emerging from large-scale genome analyses is that prokaryotes express ion channels belonging to molecular families long studied in neurons. Bacteria and archaea are now known to carry genes for potassium channels of the voltage-gated, inward rectifier and calcium-activated classes[1–3], ClC-type chloride channels[4], an ionotropic glutamate receptor[5] and a sodium channel[6]. For two potassium channels and a chloride channel, these homologues have provided a means to direct structure determination[3,7–9]. And yet the purposes of these

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