

Differential expression of two cytochrome P450 genes in compatible and incompatible Hessian fly/wheat interactions

Omprakash Mittapalli^{a,b}, Jonathan J. Neal^a, Richard H. Shukle^{a,b,*}

^aDepartment of Entomology, Purdue University, West Lafayette, IN, USA

^bUSDA-ARS, Department of Entomology, Purdue University, West Lafayette, IN, USA

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Abstract

We have recovered two Hessian fly cytochrome P450 cDNAs from an ongoing midgut EST project. *CYP6AZ1* and *CYP6BA1* represent two new subfamilies within the CYP6 family. The deduced amino acid sequences for *CYP6AZ1* and *CYP6BA1* show conserved structural and functional domains of insect P450s. Expression analysis with reverse transcription-polymerase chain reaction (RT-PCR) indicated that *CYP6AZ1* is midgut specific and induced during active larval feeding, whereas *CYP6BA1* was expressed in all tissues and developmental stages examined. Further expression analysis of *CYP6AZ1* with RT-PCR in compatible and incompatible Hessian fly/wheat interactions suggested that *CYP6AZ1* may be required for larval feeding in compatible interactions. These results should lead to a better understanding of the Hessian fly/wheat interaction with emphasis on the larval midgut as a critical interface with its host plant.

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1. Introduction

The Hessian fly, *Mayetiola destructor*, is the most destructive insect pest of wheat worldwide including the US. Effective control has been via deployment of resistant cultivars (Gallun, 1977). Emergence of virulent biotypes to resistance genes remains a threat for long-term protection (Martin-Sanchez et al., 2003). Thus, there is a need for a better understanding of the interaction between the Hessian fly and its host plant.

In a compatible Hessian fly/wheat interaction, larvae are virulent and wheat plants are susceptible. Characteristic features of first instar larvae in a compatible interaction include establishment of a feeding site and

successful completion of three instars near the crown of seedling plants, or just above the nodes of older jointed wheat plants (Shukle, 2003). Systemic symptoms in susceptible wheat plants include increased leaf sheath permeability (Shukle et al., 1992), stunting of the plant, chloroplast accumulation leading to the development of a dark green color plant and ultimately death of seedling plants (Cartwright et al., 1959). Even a single virulent larva per plant is sufficient to produce the above-mentioned symptoms, which may be caused by the secretion of unknown substances from the salivary glands (Byers and Gallun, 1972; Stuart and Hatchett, 1988).

In an incompatible interaction, larvae are avirulent and wheat plants are resistant. First instar larvae fail to establish a feeding site, larval growth and development is retarded and larvae die within a period of 2–5 days of hatching (Painter, 1930). Wheat plants participating in an incompatible interaction undergo no or little physiological stress or yield loss, and develop into

*Corresponding author. Department of Entomology, Purdue University, West Lafayette, IN, USA. Tel.: +1 765 494 6351; fax: +1 765 496 1219.

E-mail addresses: omitt@purdue.edu (O. Mittapalli), shukle@purdue.edu (R.H. Shukle).

normal mature plants (Williams et al., 2002). However, it has been found that feeding by virulent larvae can lead to the breakdown of resistance in wheat plants and allow both virulent and avirulent larvae to feed, develop, and mature (Grover et al., 1989).

We are interested in genes that may influence compatible and incompatible interactions. Among the expressed sequence tags (ESTs) we have identified from first instar Hessian fly midgut are two cytochrome P450 genes. Cytochrome P450s and esterases that are typically present in the midgut of insects are important components of the allelochemical detoxification systems in insect herbivores. Multiple P450 families have been found in insects including CYP6 (<http://drnelson.utmem.edu/CytochromeP450.html>). Cytochrome P450 genes of the CYP6 family have been reported in other insects to be involved in metabolism of plant defensive chemicals (Scott et al., 1998; Kahn and Durst, 2000) and insecticides (Feyereisen, 1999; Scott, 1999; Scott and Wen, 2001).

This paper presents the first reports of cloned genes encoding cytochrome P450 enzymes in the Hessian fly. Our interest in the cytochrome P450 enzymes expression profile of Hessian fly larvae stems from their possible involvement in the gene-for-gene interaction that determines host resistance in wheat and, more importantly, in the damage caused to the host plant by feeding larvae. In this paper, we report the cDNA sequence and gene expression of two cytochrome P450 genes recovered from a cDNA library of the Hessian fly larval midgut. The deduced amino acid sequences include several conserved amino acid residues that are characteristic of other insect CYP6 P450s. We demonstrate that *CYP6AZ1* is associated with feeding and not induced in incompatible interactions. *CYP6BA1* was constitutively expressed. This study is intended as a basis for future studies on the Hessian fly midgut as an interface with its host plant.

2. Materials and methods

2.1. Experimental insect and plant material

Hessian fly biotypes, Great Plains (GP), Biotype L and vH9 were used in this study. Sixteen biotypes of Hessian fly (designated GP and A–O) have been identified (Ratcliffe et al., 1994). Biotype GP was reared on susceptible wheat seedlings (cv. 'Newton') carrying no resistance genes in growth chambers at 20 °C with a 12-h photoperiod. The laboratory culture of GP was established from a field collection made in Ellis County, Kansas. Biotype L was selected from a field collection made from Posey county, Indiana in 1986. Biotype vH9 was selected from a field collection made from McMillan county, Georgia in 2000. Both biotypes

(Biotype L and vH9) were selected according to the methods described by Sosa and Gallun (1973). Larvae (1st-, 2nd-, and 3rd instars) and pupae were collected by dissecting the crown portions of infested wheat seedlings immersed in water and immediately flash-frozen in liquid nitrogen. Adults were collected after emergence, cold anesthetized, and flash-frozen in liquid nitrogen. Larvae, pupae, and adults were stored at –70 °C until RNA was isolated.

2.2. Salivary gland, midgut dissections, and RNA isolation

About 300 midguts were dissected from late 1st-instar larvae (5 days old) and early 2nd-instar larvae (8 days old) immersed in ice-cold Schneider's insect medium (Sigma-Aldrich). Midguts were removed by first pinching off the posterior tip of a larva and then gently compressing the body, commencing from the anterior end, resulting in the expulsion of the gut (Grover et al., 1988). Salivary glands from late 1st instars and early 2nd instars were dissected in Schneider's insect medium by grasping the posterior end of a larva with a pair of forceps while a second pair of forceps was used to grasp and pull away the anterior tip of the larva with salivary glands attached to the mouthparts. The salivary glands were then removed from connecting tissues. Midguts, salivary glands, and the remaining larval carcasses after removal of midguts and salivary glands were collected in 100 µl of ice-cold Schneider's contained in a 1.5 ml Eppendorf micro-centrifuge tube. Immediately following collection, midguts, salivary glands, and carcasses were flash-frozen in liquid nitrogen and stored at –70 °C until RNA was isolated. Total RNA was extracted from whole larvae and isolated tissues using the RNAqueous[®]-4PCR kit from Ambion following the manufacturer's protocol.

2.3. Construction of midgut cDNA libraries

Total RNA was isolated from all 300 midguts using the RNAqueous[®]-4PCR kit from Ambion. A cDNA library was constructed from the pooled RNA using a 'SMART[™]' cDNA library construction kit from BD Biosciences Clontech (Palo Alto, CA, USA) following the manufacturer's protocol with one alteration. The polymerase chain reaction (PCR) fragments obtained were cloned directly into the PCR[®]4-TOPO[®] vector included in a TOPO TA cloning[®] for sequencing kit (Invitrogen, Carlsbad, CA, USA) rather than cloning into the original phage vector. Plasmid DNA was isolated using a Qiagen BioRobot 3000 and sequenced from both ends using T7 forward, T3 reverse, and sequence-specific primers in an ABI 3700 DNA analyzer.

2.4. Characterization of the genomic sequences for the two Hessian fly P450s

Genomic clones for the coding regions of the P450s were recovered by gene-specific PCR using DNA isolated from Biotype GP adults as the template. PCR primers designed to the ends of the coding regions used to amplify the cDNA and genomic sequences were:

CYP6AZ1F,

5'-ATGTTTATCAAATTTGCTTTTTGC-3';

CYP6AZ1R,

5'-TCAATGCCTGTACGTGGCTTTCAGT-3';

CYP6BA1F,

5'-ATGTTTCGTTTTATATTTATTGA-3';

CYP6BA1R,

5'-TCACAGAATGATATCGAACTCCAG-3'.

PCR was performed in a 50- μ l reaction volume using Invitrogen Platinum *Taq* DNA Polymerase High Fidelity. In each reaction, the amount of DNA and primer used was 20 ng and 10 pmol, respectively. PCR amplification included 40 cycles of 30 s denaturing at 94 °C, 30 s annealing at 55 °C, and 1 min extension at 72 °C in a PTC-Dyad DiscipleTM thermocycler (Reno, NV). PCR products were separated on 1% agarose gels, stained with ethidium bromide (0.5 μ g/ml). DNA bands were photographed and band intensity was determined using a UVP BioDoc-ItTM system (Bioimaging systems, Upland, CA).

All the PCR fragments obtained from DNA-based PCR were excised from 1% agarose gels and purified using the MinEluteTM Gel Extraction kit (Qiagen) following the manufacturer's protocol. The fragments were ligated into the blunt-end vector pSTBLUE (Novagen, Madison, WI). Three independent clones of each DNA or cDNA fragment were sequenced three times using both strands by the Purdue Genomic Center. T7 and SP6 primers and synthesized oligonucleotides to sequence internal sequences were used. Results for each clone were aligned and a consensus formed using the SeqWeb sequence analysis program (<http://silverjack.genomics.purdue.edu>) to correct sequencing errors or errors introduced by the *Taq* polymerase. Sequence similarity and annotations were done using different BLAST programs on the National Center for Biotechnology Information (Bethesda, MD) website (<http://www.ncbi.nlm.nih.gov/>).

2.5. Expression of *CYP6AZ1* and *CYP6BA1* mRNA in larval tissues and during development

The larval tissues collected from 1st and early 2nd instars as described in Section 2.2, were pooled into three categories, midgut, salivary, and larval carcasses, and the RNA extracted from each pool was used to perform the expression analysis. Reverse transcription-polymerase chain reaction (RT-PCR) to assess expression of *CYP6AZ1* and *CYP6BA1*-II mRNA in larval tissues was carried out using the SuperScriptTM One Step RT-PCR System with Platinum[®] *Taq* DNA polymerase from Invitrogen (Carlsbad, CA, USA). Each reaction was performed three times using 0.5 μ g of RNA. The RNA was initially denatured at 94 °C for 30 min. For *CYP6AZ1* expression analysis, the PCR amplification included 40 cycles of 30 s denaturing at 94 °C, 30 s annealing at 47 °C, and 1 min extension at 72 °C in a PTC-Dyad DiscipleTM thermocycler (Reno, NV). Similar conditions of PCR amplification were undertaken for the expression analysis of *CYP6BA1*, except the annealing temperature used was 58 °C. PCR products (5 μ l/reaction) were separated on 1% agarose gels, stained with ethidium bromide (0.5 μ g/ml). DNA bands were photographed and band intensity was determined using a UVP BioDoc-ItTM system (Bioimaging systems, Upland, CA).

RT-PCR to assess expression of *CYP6AZ1* and *CYP6BA1* mRNA at different times of larval development and in pupae and adults was performed as described for the larval tissues using RNA extracted from 1st instars (4 days old), early 2nd instars (8 days old), late 2nd instars (23 days old), 3rd instars (from puparia), pupae, and adults. PCR primers used in the expression analysis were:

CYP6AZ1-f,

5'-CTTGAAAAATGGTGTCAGAAATCTG-3';

CYP6AZ1-r,

5'-TCAATGCCTGTACGTGGCTTTCAGT-3';

CYP6BA1-f,

5'-ATGTTTCGTTTTATATTTATTGA-3';

CYP6BA1-r,

5'-CACCAATTAACGAATCCGATTTATG-3'.

Hessian fly actin transcript was RT-PCR amplified as a reference control using primers: Act-F, 5'-ATGTGT-GACGACGAAGTTGCTTTGGTT-3'; Act-R, 5'-CATAACGATGTTAGCGTACAAGTCCTTACG-3'. RT-PCR with RNA without reverse transcriptase to generate single stranded DNA (ssDNA) template was used as the negative control to test for possible contaminating DNA in the isolated RNA.

2.6. RT-PCR analysis of *CYP6AZ1* mRNA expression in compatible vs. incompatible interactions

To study the expression profile of *CYP6AZ1* in compatible and incompatible interactions, first instar larvae of Biotype L were collected from susceptible wheat seedlings (cv. 'Newton') and resistant wheat seedlings (cv. 'Iris') carrying the *H9* gene for resistance, which represent a compatible and an incompatible interaction, respectively. Biotype vH9 carrying virulence gene, *vH9* was also collected from 'Newton' and 'Iris' wheat seedlings that represent compatible interactions. Hatched larvae that were 1–4 days old were collected as described above for all interaction types. Larvae were collected up to day 4 for both compatible and incompatible interactions as they die within 5 days after hatching in the latter interaction (Painter, 1930). Different numbers of RT-PCR amplification cycles (i.e., 20, 25, 30, and 35 cycles) were used to assess whether amplifications had been taken to saturation. Each reaction was performed three times using equal amounts of RNA (0.5 µg) and also ssDNA samples that were normalized with a radio-labeled tracer technique. The RNA was initially denatured at 94 °C for 30 min. PCR amplification conditions included 30 s denaturing at 94 °C, 30 s annealing at 47 °C, and 1 min extension at 72 °C in a PTC-Dyad Disciple™ thermocycler (Reno, NV). An aliquot of 5 µl of the PCR reaction was assessed on 1% agarose gel after 20, 25, 30, and 35 cycles of PCR amplification. PCR products were separated on 1% agarose gels, stained with ethidium bromide (0.5 µg/ml). DNA bands were photographed and band intensity was determined using a UVP BioDoc-It™ system (Bioimaging systems, Upland, CA). Controls used were the same as described in the above sections.

2.7. Normalization of RNA samples with the radio-labeled tracer technique

Quantification of RNA using the radio-labeled tracer technique was done according to Spangler et al. (2003) and Puthoff et al. (2003) with modifications. In all, 4.35 µg of total RNA in 10 µl of water was treated with DNase using the DNase free kit from Ambion (Austin, TX) following the manufacturer's protocol. The RT reaction was carried out using Superscript First Strand Synthesis kit for RT-PCR from Invitrogen (Carlsbad, CA). In all, 1 µl of oligo d(T) primer and 1 µl of dNTPs were added to the 10 µl sample of total RNA that had been treated with DNase. The mixture was heated at 65 °C for 5 min and then put on ice. RT reactions were performed by adding the following on ice: 2 µl 10 × first strand buffer, 2 µl 50 mM MgCl₂, 2 µl 0.1 M DTT, 1 µl RNase Out, and 1 µl SuperScript II. From each reaction, an aliquot of 5 µl was taken and mixed with

1 µl pf ³²P-dCTP (Amersham, Piscataway, NJ) diluted 1:5 (³²P:water). This step was done so that each reaction had the same amount of time to incorporate ³²P. The remaining 15 µl of the RT reaction and the 5 µl samples containing the isotope were incubated at 42 °C for at least an hour. Reactions were stopped by heating samples to 70 °C for 15 min. After cDNA synthesis was complete, the 15 µl reactions were placed on ice, while the ³²P reactions were used to measure the amount of synthesized cDNA in each sample. Using the ³²P monitored reaction as a guide, the reactions pertaining to the first strand cDNA were diluted to ensure equal amounts of cDNA (10 ng of total RNA/PCR reaction) was placed in each PCR reaction.

3. Results

3.1. Nucleotide and deduced amino acid sequences

The two Hessian fly cytochrome P450s, *CYP6AZ1* and *CYP6BA1*, recovered from an ongoing midgut EST project of the Hessian fly were full-length cDNAs with open reading frames of 1689 and 1569 bases, respectively. The coding regions coded for 521 residues of *CYP6AZ1* and 523 residues for *CYP6BA1*. These deduced amino acid sequence lengths are within the range of lengths of others in the CYP6 family (Feyereisen, 1999). Genomic sequences for both P450s were obtained by PCR using genomic DNA as template and primers that were designed to amplify cDNA sequences. The differential sizes of the cDNAs and genomic DNA-PCR products revealed the presence of introns. *CYP6AZ1* coding sequence contained two introns, tentatively numbered intron I and II whereas, *CYP6BA1* coding sequence contained three introns numbered I, II, and III. For *CYP6AZ1*, intron I was 60 bases long and located near the 5' end of the gene. Intron II was 68 bases and located near the 3' end of the gene. For *CYP6BA1*, intron I was 55 bases long and located near the middle region of the gene, while intron II (96 bases) and intron III (94 bases) were found near the 3' end of the genomic sequence. All five introns pertaining to both P450 genomic sequences obeyed the GT–AG rule (Lewin, 2002). No introns were identified within the 5' or 3' un-translated regions for either *CYP6AZ1* or *CYP6BA1*. A putative polyadenylation signal, AATAAA, which matches with other species (Tomita and Scott, 1995), is present 55 and 20 bp downstream of the stop codon in *CYP6AZ1* and *CYP6BA1* sequences, respectively. The nucleotide sequences of *CYP6AZ1* and *CYP6BA1* were submitted to GenBank and have the following GenBank accession numbers AY884043 and AY884044, respectively.

Both of the Hessian fly P450 genes, *CYP6AZ1* and *CYP6BA1*, represent new subfamilies in the CYP6

family according to the nomenclature system followed for P450s classification (Nelson, 2004, pers. comm.). The Hessian fly P450 sequences were compared with other Dipteran CYP6 members to reveal functional or conserved regions in the former. CYP6AZ1 and CYP6BA1 have the sequence FXXGXXXCXG located toward their C terminus of the deduced amino acid sequence, which is commonly referred to as a “signature” of all P450 genes and thus is conserved in all the members of the P450 superfamily (Schuler, 1996; Feyereisen, 1999). The signature sequences for CYP6AZ1 and CYP6BA1 are FGDGARNICG and FGDGPRSCIG, respectively. The cysteine residue in this conserved sequence is a characteristic feature of classical P450s and represents the fifth ligand to the heme iron (Gotoh et al., 1983; Feyereisen, 1999).

In order to determine conserved domains in CYP6AZ1 and CYP6BA1 deduced amino acid sequences, the BLAST program was used (Altschul et al., 1990). The results from this search engine indicated that CYP6AZ1 exhibits greatest similarity with *Culex pipiens quinquefasciatus* CYP6F1 (BAA92152), *Drosophila simulans* (AAR36858), *Drosophila melanogaster* CYP6A2 (AAM70832), and *Aedes aegypti* CYP6AL1 (AAV41248). CYP6BA1 exhibits greatest similarity with *Anopheles gambiae* CYP6Z3 (AAO24698), *D. melanogaster* CYP6D5 (AAM48328), *C. pipiens quinquefasciatus* CYP6F1 (BAA92152), and *Musca domestica* CYP6D1 (AAC99340). A peptide alignment was made to identify conserved amino acids in CYP6AZ1 and CYP6BA1 (Fig. 1). This analysis included the corresponding Hessian fly cytochrome P450 and its four most similar sequences. The obtained alignment indicated 76 and 97 identical amino acid residue positions for CYP6AZ1 and CYP6BA1, with the four sequences included in the alignment (Fig. 1). Amino acid identity of CYP6AZ1 with CYP6F1, CYP6A26, CYP6A2, and CYP6AL1 was 43%, 39%, 39%, and 38%, respectively. For CYP6BA1, the amino acid identity with CYP6Z3, CYP6D5, CYP6F1, and CYP6D1 was 41%, 39%, 38%, and 37%, respectively.

3.2. Expression of CYP6AZ1 and CYP6BA1 in larval tissues and during development

RT-PCR analysis revealed that CYP6AZ1 was expressed only in the midgut tissue and not in the salivary glands and the remaining carcasses tissues. However, CYP6BA1 was expressed in all tissues including the midgut, salivary glands, and remaining carcasses (Fig. 2). RT-PCR analysis for different developmental stages indicated that CYP6AZ1 was expressed only in feeding instars (1st instars, and early and late 2nd instars) whereas CYP6BA1 was expressed in all the developmental stages including the feeding instars, non-feeding 3rd instars, pupae, and adults

(Fig. 3). These results suggest a relationship of CYP6AZ1 to larval feeding.

3.3. Differential expression of CYP6AZ1 in compatible and incompatible Hessian fly wheat interactions

The expression profile of CYP6AZ1 in virulent and avirulent larvae was assessed with 20, 25, 30, and 35 RT-PCR amplification cycles. CYP6AZ1 was expressed in all three compatible interactions following 20 cycles of PCR amplification; however, there was no expression of CYP6AZ1 detected with the same number of PCR cycles with RNA from incompatible interactions (data not shown). Differential expression of CYP6AZ1 was most prominent with RNA from the interactions following 25 cycles of PCR amplification (Fig. 4). Expression of CYP6AZ1 was much higher in compatible interactions when compared to its expression in incompatible interactions after 25 cycles of PCR amplification. Even after 30 cycles of PCR amplification, the increased expression trend of CYP6AZ1 mRNA in compatible interactions was evident. However, saturation for amplification of the transcript was observed in both interactions following 35 cycles of PCR amplification. Primers designed to amplify a 101 bp fragment of a Hessian fly actin was used as an internal control. Amplification for the actin was detected following 15 cycles of PCR amplification (data not shown) and approached saturation with 25 cycles. RNA extracted from non-infested plants was used as a negative control, and no amplicons were detected after 35 cycles of PCR amplification. These results combined with the observations in Section 3.3 (expression of CYP6AZ1 and CYP6BA1 in larval tissues and during development) suggest that expression of CYP6AZ1 is induced during feeding and may have a role in the survivability of virulent larvae participating in compatible interactions.

4. Discussion

Results with RT-PCR for Hessian fly CYP6AZ1 in different tissues and developmental stages indicated that it was midgut specific in expression (Fig. 2) and was induced only in the feeding instars (Fig. 3). Several studies have demonstrated the midgut of insects to be one of the major organs involved in detoxification of allelochemicals (Cohen et al., 1992; Cariño et al., 1994; Snyder et al., 1995). An initial report on the detoxification role of cytochrome P450s, which is probably the best example studied thus far in an insect–plant interaction, was demonstrated in swallowtails (*Papilio* sp.), wherein furanocoumarins present in swallowtail larval midguts were significantly detoxified by CYP6B1 (Cohen et al., 1992). The CYP6B1-based detoxification

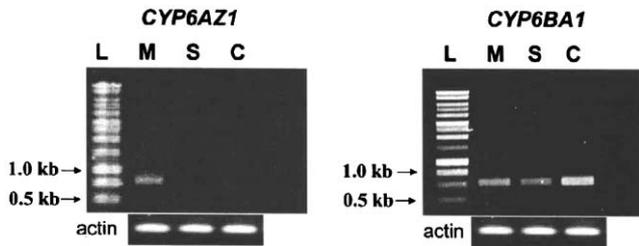


Fig. 2. RT-PCR analysis of *CYP6AZ1* and *CYP6BA1* with RNA from different tissues of Hessian fly larvae. A PCR product was detected for *CYP6AZ1* with RNA from midgut tissue (Lane M). No PCR product for *CYP6AZ1* was detected with RNA from salivary glands (Lane S) or larval carcasses (Lane C). A PCR product was detected for *CYP6BA1* with RNA from midgut tissue (Lane M), salivary glands (Lane S), and larval carcasses (Lane C). In both the panels shown, 5 µl of each PCR (50-µl reaction volume) was loaded per lane. A Promega 1 kb DNA ladder was run in Lane 1. RT-PCR product obtained with primers designed to a Hessian fly actin gene and RNA extracted from midgut tissue (Lane 2), salivary glands (Lane 3), and remaining larval carcasses (Lane 4) is shown as an insert.

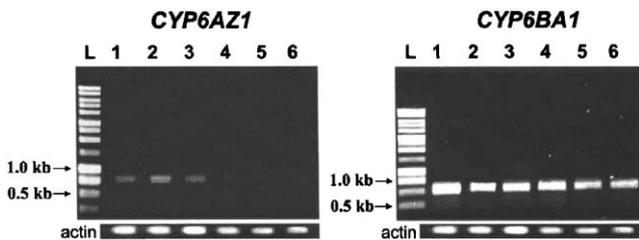


Fig. 3. RT-PCR analysis of *CYP6AZ1* and *CYP6BA1* with RNA from different life stages of the Hessian fly. A PCR product was detected for *CYP6AZ1* with RNA from 1st instars (Lane 2), early 2nd instars (Lane 3), and late 2nd instars (Lane 4). No PCR product was detected for *CYP6AZ1* with RNA from 3rd instars (Lane 5), pupae (Lane 6), or adults (Lane 7). A PCR product was detected for *CYP6BA1* with RNA from all the stages including 3rd instars (Lane 5), pupae (Lane 6), and adults (Lane 7). In both panels shown, 5 µl of each PCR (50-µl reaction volume) was loaded per lane. A Promega 1 kb DNA ladder was run in Lane 1. RT-PCR product obtained with primers designed to a Hessian fly actin gene and RNA extracted from 1st instars (Lane 2), early 2nd instars (Lane 3), late 2nd instars (Lane 4), 3rd instars (Lane 5), pupae (Lane 6), and adults (Lane 7) is shown as an insert.

enzymes in herbivorous insects during larval feeding has been associated with their inherent capability to detoxify a wide range of plant defense toxins (allelochemicals). This remarkable function includes overlapping substrate specificity, and is determined by the presence of a suite of cytochrome P450 molecules, a major constituent of the cytochrome P450 enzyme system (Tomita and Scott, 1995). This relationship is critical for survival (Schuler, 1996; Feyereisen, 1999; Li et al., 2004). The results obtained in this study pertaining to expression of *CYP6AZ1* combined with the observations pertaining

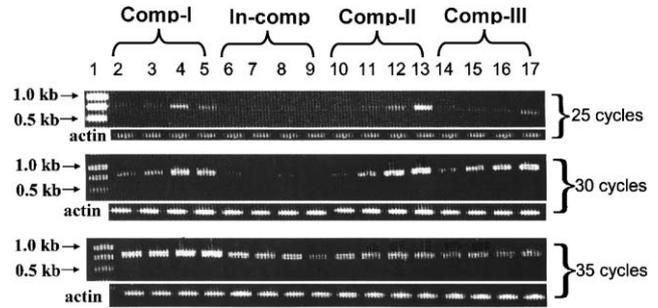


Fig. 4. RT-PCR analysis of *CYP6AZ1* in compatible (comp) and incompatible (incomp) Hessian fly/wheat interactions. Lanes 2–5 represent a compatible interaction involving Biotype vH9 on ‘Iris’ with RNA collected from 1 to 4 days old larvae. Lanes 6–9 represent an incompatible interaction involving Biotype L on ‘Iris’ with RNA collected from 1 to 4 days old larvae. Lanes 10–13 represent a compatible interaction involving Biotype L on Newton with RNA collected from 1 to 4 days old larvae. Lanes 14–17 represent a compatible interaction involving Biotype vH9 on Newton with RNA collected from 1 to 4 days old larvae, respectively. PCR products were detected for all compatible interactions from 20 cycles of amplification (data not shown). PCR products were not detected for incompatible interactions at 25 cycles, while PCR products not approaching saturation were detected with 30 cycles of amplification. In all, 5 µl of each PCR (50-µl reaction volume) was loaded per lane. A Promega 1 kb DNA ladder was run in Lane 1. RT-PCR product obtained with primers designed to a Hessian fly actin gene used as an internal control for each RT-PCR reaction and is shown as an insert.

to its midgut specific expression, strongly indicate that expression of *CYP6AZ1* is induced during feeding and thus may have an important role in the survivability of virulent larvae participating in compatible interactions and may metabolize allelochemicals from the wheat plants.

One interpretation of the expression pattern of *CYP6AZ1* is that it is induced by substances ingested from the plant. There are numerous accounts of P450 expression or induction in response to dietary allelochemicals, including *Depressaria pastinacella CYP6AB3* (Li et al., 2004), *Papilio glaucus CYP6B4* (McDonnell et al., 2004), *Helicoverpa zea CYP6B* and *CYP321A1* (Sasabe et al., 2004), and *Papilio polyxenes CYP6B1* and *CYP6B3* (Harrison et al., 2001); all inducible by dietary furanocoumarins. Cytochrome P450s may be induced by numerous other plant allelochemicals including jasmonate and salicylate (Li et al., 2002a) that are inducers of several *H. zea* cytochrome P450 genes. The *H. zea CYP6B9* (Li et al., 2002b) is constitutively expressed only in the midgut and inducible by plant allelochemicals. The expression of *CYP6AZ1* only in the midgut and induced during active feeding is similar to the expression pattern of *CYP6B9*.

There are several potential P450 inducers present in wheat. These include cyclic hydroxamic acids and related benzoxazolinones, an important group of allelochemicals present in Gramineae members (wheat, rye, maize, etc.). These hydroxamic acids have been

shown to be involved in plant defense against fungi, bacteria, and insects (Fomsgaard et al., 2004; Frey et al., 1997). *CYP6B* members have not only been reported to respond to a wide range of allelochemicals present in host plants (indole-3-carbinol, jasmonate, xanthotoxin, etc.) but also to synthetic chemicals, such as insecticides (Li et al., 2002a,b). Thus, possible substrate(s) for *CYP6AZ1* could involve some of the above-mentioned substrates or other allelochemicals that exist in wheat.

The expression profile of Hessian fly *CYP6BA1* was different from that of *CYP6AZ1*. *CYP6BA1* transcripts were detected in all tissues (Fig. 2) and developmental stages examined (Fig. 3). Recently, Yoshiyama and Shukle (2004) observed the expression of a GST (*mdesgst-1*) in all tissues of Hessian fly larvae including salivary glands, fat body and midgut tissue, and suggested a biochemical defensive role in the Hessian fly/wheat interaction. *CYP6BA1* could be participating in detoxification in salivary glands, fat body, and midgut tissue thereby contributing in the biochemical adaptation of the Hessian fly larvae to wheat or could also be important in performing other tasks, such as biosynthetic pathways. The selective induction of *CYP6AZ1* compared to the constitutive expression of *CYP6BA1* probably indicates that cytochrome P450 expression in the Hessian fly is influenced by chemicals present in the host plant. These results support observations made by Snyder et al. (1995), who found that specific inducers added to the diet could cause a selective expression of *Manduca sexta* CYP4 genes.

Cytochrome P450s may have an important role in establishing compatible interactions and host plant resistance. The mode of resistance offered by the host plant against Hessian fly is governed predominantly by single genes that are completely to partially dominant (Zantoko and Shukle, 1997). This mode of resistance is expressed as larval antibiosis (El Bouhssini et al., 1998). Genetic analysis suggests that for each resistance gene in a host there is the presence of a corresponding avirulence gene in a pathogen, which forms the basis for a gene-for-gene interaction (Flor, 1971). The interaction between the Hessian fly and wheat is very specific. The resistance mechanism in wheat plants is triggered only when the resistant plant can recognize elicitors secreted by a feeding larva of an avirulent biotype (Williams et al., 2002). However, recent studies have indicated that the gene-for-gene interaction in the Hessian fly/wheat interaction is not as vivid as thought earlier but rather more complex (El Bouhssini et al., 1998).

Further studies directed toward learning the functionality of both the Hessian fly P450 genes, in particular *CYP6AZ1*, via double-stranded mediated RNA interference (RNAi) could provide a better experimental dissection of the Hessian fly/wheat interaction. Additionally, recovery of the promoter sequence and more

upstream regions of the *CYP6AZ1* gene could reveal the regulatory elements that affect its expression in compatible and incompatible interactions.

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