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Two differentially expressed ommochrome-binding protein-like genes (*obp*1 and *obp*2) in larval fat body of the European corn borer, *Ostrinia nubilalis*

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Abstract

Ommochrome-binding proteins function in coloration and detoxification pathways by transporting tryptophan metabolites, and increase in hemolymph concentration prior to diapause. Two ommochrome-binding protein genes from the European corn borer *Ostrinia nubilalis* (Hübner) (On*obp*1 and On*obp*2; GenBank accession nos. AY819651 to AY819655 and AY862870) were isolated. Relatedness to OBP-encoding genes was suggested by peptide similarity, phylogenetic reconstruction, and expression data. 21 single nucleotide polymorphisms between *obp*1 and 23 polymorphisms between *obp*2 alleles were identified, and resultant genomic markers were inherited in a Mendelian fashion. RT-PCR showed fat body specific On*obp*1 and On*obp*2 transcription. The On*obp*1 transcript was RT-PCR amplified from fat body of 5th instars, whereas On*obp*2 was expressed in fat body of 4th and 5th instars, and peaked in 5th instar wandering and 1 week old diapausing larvae. Expression suggests gene duplicates are maintained by change in temporal expression. The significance of On*obp*1 and 2 gene products to *O. nubilalis* diapause physiology requires additional investigation.

Keywords: Ostrinia nubilalis, diapause induction, transcriptional variation

<u>*Abbreviation:*</u>

DAP	diapause-associated polypeptide
OBP	ommochrome binding protein
On*obp*1	*Ostrinia nubilalis* ommochrome binding protein 1
On*obp*2	*Ostrinia nubilalis* ommochrome binding protein 2

Introduction

Ommochromes (ommatin and ommin) are tryptophan derivatives (Kayser 1985) biosynthesized from kynurenine and 3-hydroxykynurenine, and noticed as yellows, red, brown, and black pigments (Oxford and Gillespie 1998). These pigments are localized in eye and cuticle, and excreted into the gut lumen as part of a detoxification pathway (Linzen 1974). Insects lack analogous vertebrate glutarate (Linzen 1974) and nicotinic acid pathways (Kayser 1985), and thus rely on conversion of tryptophan to ommochromes for removal. Ommochrome binding proteins (OBPs) transport these pigments in Lepidoptera through the hemolymph within a general detoxification pathway (Martel and Law 1991; 1992). OBPs are 31 kDa glycoproteins that were characterized from *Manduca sexta* (Martel and Law 1991; Yepiz-Plascencia *et al*. 1993), and *Antheraea yamamai* (Saito *et al*. 1998). OBP level in the hemolymph increase significantly during late instars (Martel and Law 1992) and may serve as a marker of lepidopteran diapause induction. Larval diapause associated polypeptides (DAPs) also show increased hemolymph levels during late instars and function in nutritive storage (Palli *et al*. 1993).

The European corn borer, *Ostrinia nubilalis* (Hübner), is a crop pest in its larval stage. Voltinism ecotypes of *O. nubilalis* (uni-, bi-, and multivoltine) show differing larval response to photoperiod and temperature for induction of diapause (Showers 1993; Mason *et al*. 1996). Additionally, phenotypic differences in number of degree-days required post-diapause prior to pupation were observed between voltine ecotypes (Showers 1979), and showed paternal inheritance (Showers *et al*. 1972; Reed *et al*. 1981; Showers 1981). Juvenile hormone may play a major role in control of ecdysis, diapause maintenance, and metamorphosis (Yin and GM Chippendale 1976), and suppression of 20-hydroxyecdysone is crucial in delay of molting during diapause (Denlinger 1985). The diapause state also is characterized by brain inactivity, and non-response of the prothoracic glands to activation by the prothoracicotropic hormone (Richard and Saunders 1986; Gelman *et al*. 1992). Larval diapause induction is marked by physiological changes in hemolymph composition (Chippendale and Beck 1967).

Harsh winter conditions are natural barriers to pest species range expansion. The ability of a species or ecotype to enter and maintain diapause may dictate survival in geographic ranges by delaying reproductive cycles until more favorable climatic conditions occur. North-south clines are observed with univoltine *O. nubilalis* ecotypes restricted to northern ranges (Showers 1979; Showers 1993). The univoltine *O. nubilalis* ecotype may have selective advantages in northern climates where short growing seasons favor single generations (Showers 1993). An extended time is required for univoltine larvae to break diapause compared to bivoltine counterparts (Calvin and Song 1994; Hoard and Weiss 1995), suggesting genes or environment influence voltinism traits. Physiological changes associated with *O. nubilalis* larval diapause induction are manifested in the hemolymph (Chippendale and Beck 1967). We focused on characterization of ommochrome binding proteins from *O. nubilalis* as molecular markers of diapause, and to better understand genes expressed at the onset of the diapause state. Genetic markers developed herein also can be integrated into a molecular linkage map of the *O. nubilalis* genome.

Materials and Methods

Ostrinia nubilalis samples and extraction protocols

Fat body, midgut, head, and whole body samples were collected from 5th instar *O. nubilalis* larvae. Additionally, fat body tissue was removed from 4th and 5th *O. nubilalis* larvae, and from larvae exposed to diapause conditions for one week (Beck 1982). All *O. nubilalis* were bivoltine Z-pheromone strain individuals from the USDA-ARS, Corn Insects and Crop Genetics Research Unit lab colony in Ames, Iowa. Dissected tissue samples were flash frozen in liquid nitrogen, ground to powder, and RNA extracted with RNeasy extraction kits (Qiagen, www.qiagen.com) according to manufacturer instructions. All DNA extractions used adult thoracic tissue and were performed as described by Coates and Hellmich (2003). Extracts were quantified by absorption at 260 nm (A^{260}), diluted to 50 ng/μl, and stored at –20°C.

PCR primer design, DNA amplification, and sequencing

Forward (DAP1-F: 5´-TTA GCG AGT GCT GCC TTG GT-3´) and reverse PCR primers (DAP1-R: 5´-GTC AGG CGC ATC ACA CTG TT-3´) were designed from an *O. furnacalis* diapause-associated protein gene sequence (GenBank accession no. AF169311) using Primer3 (Rozen and Skaletsky 1998). Primers spanned the coding sequence from amino acid positions 10 through 269 of the 291 amino acid long *O. furnacalis* gene. The homologous *O. nubilalis* gene region was PCR amplified using 5 pmol of each primer in 2.5 mM $MgCl_2$, 50 μM dNTPs, 100 ng of DNA template, and 0.9 U *Taq* DNA polymerase (Promega, www.promega.com) in a 12.5 μl reaction. PTC-100 thermocycler conditions used 95° C for 2.5 m, followed by 40 cycles of 95° C for 30 s, 58° C for 30 s, and 72° C for 1 m. Amplification was confirmed and fragment size estimated by electrophoresis of 6 μl PCR product on a 10 cm 1.5 % agarose 1X tris-borate EDTA (1X TBE) gel containing 0.5 μg/ml ethidium bromide. PCR product was ligated using the pGEM-T Easy Cloning system (Promega) by overnight incubation at 4° C, and was used to transform 80 μl *E. coli* SURE (Stratagene, www.stratagene.com). Cloned inserts were DNA sequenced in 10 μl DTCS Quickstart reactions (Beckman-Coulter, www.beckman.com) using 1.6 pmol T7 primer according to manufacturer instructions. Sequence reaction products were purified by ethanol precipitation, suspended in 40 μl deionized formamide, and separated on a CEQ 8000 Genetic Analysis System (Beckman-Coulter) with method LFR-1 (denature: 90° C for 120 sec; inject: 2.0 kV for 15 sec; and separated: 4.2 kV for 85 min in a 50° C capillary). Sequence data was used to design transcript-specific reverse primers. Electropherogram output from duplicate runs was inspected visually for sequencing errors on the CEQ8000 Genetic Analysis Software.

Gene homology and orthology

The *Ostrinia furnacalis* diapause-associated protein sequence (DAP; GenBank accession no. AF169311; positions 246 to 762) was aligned with homologous regions from three *O. nubilalis obp*1 and 2 *obp*2 alleles, and *M. sexta* OBP gene sequence (GenBank accession no. L00975; positions 59 to 575). A 350 nt On*obp*2 allele fragment (Onobp2-B4) that spanned *O. furnacalis* DAP positions 487 to 836 was omitted from the alignment. Additionally, expressed sequence tag sequences from *Bombyx mori* 5th instar fat body (fbVm022; male; strain p50) and ovary (ovS318H0, and ovS10G0) were include in the alignment. The consensus alignment was generated using AlignX software (Informax; gap penalty = 10), and a shared 516-nucleotide region of the alignment was used to construct a parsimony-based phylogeny using programs from the PHYLIP package (Felsenstein 1989). *Bombyx mori* ovarian expressed sequence tag ovS10G0 was used as the outgroup. One thousand bootstrap resampling steps were produced by the SeqBoot program, parsimony trees were generated using DNAPars, a strict consensus tree was estimated from all possible phylogenies with CONSENSE, and was viewed using TreeView (Page 1996).

Expression analysis

Total RNA extracted from fat body, midgut, head, and whole larvae from 5th instar larvae, and fat body from 4th and 5th, 5th instar wandering, and 1 week old diapausing *O. nubilalis* larvae was subjected to reverse transcriptase (RT)-PCR analysis. Individual RT-PCR first strand cDNA synthesis reactions used 250 ng total RNA template, 10 pmol of primer DAP1-R or β-actin-R (5´-GAC AAC GGC TCC GGT ATG T-3´; controls only), 2.5 U *Tth* polymerase (Promega), 100 μM dNTPs, 2.5 mM $MnCl_2$, and 2.0 μl of 10X *Tth* reverse transcriptase buffer (Promega) in a 10 μl reaction volume. A PTC-100 thermocycler (MJ Research, www.mjr.com) performed a primer extension cycle of 85° C for 1 m, 56° C for 1 m, and 72° C for 20 m. A 4.0 μl aliquot of 1st strand cDNA synthesis product was mixed with 1.6 μl chelate buffer (Promega), 10 pmol of primer OnOBP1-F, OnOBP2-F, or β-actin-F (5´-CCT TCG TAG ATA GGG ACG GT-3´; controls only), $MgCl_2$ concentration adjusted 2.0 mM in a 20 μl final volume. RT-PCR reactions were carried out on a PTC-100 thermocycler (MJ Research) using 40 cycles of 95° C for 30 s, 56° C for 30 s, and 72° C for 1 m. RT-PCR products (20 μl) were separated on a 10 cm 1.0 % agarose 1X tris-borate

EDTA gel containing 0.5 µg/ml ethidium bromide, and digital images taken under UV illumination on a BioRad ChemiDoc System (BioRad, www.bio-rad.com).

Ostrinia nubilalis ommochrome binding protein (OBP) polymorphism

Two putative *O. nubilalis* ommochrome binding protein genes, called On*obp*1 and On*obp*2, were co-amplified using primers DAP1-F and DAP1-R. Gene specific forward PCR primers OnOBP1-F (5´-GGG AGC GTG CTR AAG ACC AT-3´) and OnOBP2-F (5´-GGG ACT GTG CTG ATG AAG A-3´) were designed using Primer3 (Rozen and Skaletsky 1998) that could be used in combination with DAP1-R. The two PCR products were amplified separately, but with the same conditions (4 pmol of each primer, 2.5 mM $MgCl_2$, 50 µM dNTPs, 100 ng of DNA template, and 0.9 U *Taq* DNA polymerase (Promega) in a 10.0 µl reaction). PTC-100 thermocycler conditions used 95° C for 2.5 m, followed by 40 cycles of 95° C for 30 s, 58° C for 30 s, and 72° C for 1 m.

Polymorphic restriction endonuclease cleavage sites were identified in On*obp*1 and On*obp*2 gene fragment alignments, and used to screen pedigrees and genotype light trap samples. The On*obp*1 fragment was digested with *Hinf*I or *Tsp*509I, and the On*obp*2 fragment was digested with *Hae*III, *Mse*I or *Taq*I. All endonucleases were purchased from New England BioLabs except *Taq*I (Promega). PCR-RFLP reactions included 6.0 µl of appropriate PCR product, 2.5 µl 10x Buffer, 0.1 mg/µl BSA, and 0.25 U of enzyme in 25 µl. Reactions were incubated at 37° C or 60° C (*Tsp*509I and *Taq*I) for 14 h. Entire PCR-RFLP reaction volumes were loaded onto 10 cm 2.0 % 1X TBE agarose gels that contained 0.5 µg/ml ethidium bromide. Samples were separated at 100 V for 1.5 h, and images captured under UV illumination on a ChemiDoc System (BioRad).

Mendelian inheritance of Onobp1 and Onobp2

Mendelian inheritance of On*obp*1 and 2 was evaluated by allele segregation in pedigrees. Two paired matings of *O. nubilalis* adults from the USDA-ARS, CICGRU laboratory colony were used to establish F_1 families. The F_1 progeny were sib-mated (inbred) to produce F_2 larvae. F_2 larvae were reared on a semi-meridic diet (Guthrie 1987), DNA extracted from adults according to Coates and Hellmich (2003), and samples stored at – 20° C prior to use. On*obp*1 and On*obp*2 loci were screened by PCR-RFLP as described previously, and chi-square tests used to measure goodness-of-fit between observed allelic distributions in the F_2 generation and expectation Mendelian ratios.

Results and Discussion

Gene characterization and amplification

Two highly similar ommochrome-binding protein-like (*obp*) genes were characterized from the *O. nubilalis* genome. Both sequences were isolated from *O. nubilalis* by PCR, named On*obp*1 and On*obp*2, and the data were submitted under GenBank accession nos. AY819651 to AY819655. GenBank accession nos. AY819651 and AY819652 are partial On*obp*1, and AY819653 to AY819655 and AY862870 are partial On*obp*2 genes that lack N- and C-terminal peptide coding regions. Both *O. nubilalis* genes are transcribed in fat body tissue only, show derived structural characteristics similar to the *M. sexta* OBP, and likely encode glycoprotein products excreted into hemolymph. Structural identification of *Ostrinia* sequences as OBP encoding genes was based on peptide identity to the *M. sexta* OBP, similarity of the signal peptide, N-glycosylation, a conserved glycosyl hydrolase family 10 active site, and SwissProt classification of an *O. furnicalis* homolog within peptide Family PD125063 with the *M. sexta* OBP.

A PCR product amplified from *O. nubilalis* genomic DNA using primers DAP1-F and DAP1-R contained a heterogeneous mix of two approximately 775 bp products. The *O. nubilalis* sequences were gene fragments that omitted 9 and 22 codons from N- and C-terminal peptide regions, respectively. The *O. nubilalis* nucleotide sequences were 90-98% similar to an *O. furnacalis* diapause-associated peptide coding sequence (DAP; GenBank accession no. AF169311; Fig. 1; BLAST score ≥ 233 and E-score $\geq 6 \times 10^{-30}$). All *O. nubilalis obp*1 and 2 genes contained > 90% of the CDS length and shared ≥ 80% identity at the peptide-level compared to *O. furnacalis*. The unpublished *O. furnacalis* GenBank accession AF169311 was therefore used to establish similarity to known genes. The 291 amino acid *O. furnacalis* DAP1 peptide showed similarity (35 %) and identity (54 %) to a 274 residue (31 kDa) *M. sexta* ommochrome binding protein encoding gene (*obp*; Martel and Law 1991; Yepiz-Plascencia *et al.* 1993; GenBank accession no. L00975; BLASTp score = 134, E-value 10^{-30}). A BLASTp search of using the *M. sexta* peptide P31420 identified our released *O. nubilalis obp*1 and 2 sequences (E-values 4×10^{-14} to E-value 3×10^{-36}; identities 34 to 38%; similarities 53 to 58%). Identities as low as 25% were present between putative chymotrypsin-like proteases from the Hessian fly (*Mayetiola destructor*; Zhu 2005) even though genes were derived from a single ancestor via gene duplication (Neurath *et al.* 1967). The peptide similarity of 46% was used to identify a 269-residue *B. mori* peptide fragment derived from fat body expressed sequence tag fbVm0227 as an ommochrome binding protein (SilkBase, Bombyx Genome Database Working Group; Toru Shimada, Kazuei Mita, and co-workers unpublished). This suggests relative homology between *Ostrinia* and *M. sexta* genes is sufficient to classify the former as ommochrome-binding protein-related genes.

The derived amino acid sequence from *O. furnacalis* DAP GenBank accession no. AF169311 had a predicted molecular weight ≅ 32.6 kDa and isoelectric point (pI) of 4.89 (compute pI/MW program; Bjellqvist *et al.* 1993) that was near 31 kDa and pI of 5.89 for *M. sexta* OBP (Martel and Law 1991). The *O. furnacalis* peptide was predicted to have extracellular localization with 66.7% assurance (PSORTII program; Paul Horton unpublished; www.psort.org). Additionally, the *O. furnacalis* peptide may have an 18 amino acid signal peptide (residues 1 to 18) predicted by PSORTII program using methods described by von Heijne (1986), which is the exact length of the signal peptide from *M. sexta*. Although the two signal sequences appear to share little sequence identity, *O. furnacalis* and *M. sexta* OBP signal peptides were respectively composed of 12/18 and 11/18 residues with aliphatic side chains. Furthermore, the program SignalP 3.0 (www.cbs.dtu.dk/services/SignalP/) used a hidden Markov models to predict presence of a peptide cleavage site following a VSS residue sequence of the *O. furnicalis* OBP peptide (probability 0.998). The partial *O. nubilalis obp*1 and *obp*2 sequences contained 50% of the entire signal sequence length and is the location of forward PCR primer (DAP1-F), showing the *O. nubilalis obp*1 and *obp*2 genes likely also encode a similar signal sequence. These properties suggest cell membrane transport out of the fat body, and proteins that are soluble in lepidopteran hemolymph (pH ≅ 7.0; see expression data below).

The derived peptide sequences from *O. furnacalis* or *O. nubilalis* gene fragments did not show similarity to *Choristoneura fumiferana* (Lepidoptera: Tortricidae) DAP1 (753 amino acids; 74 kDa) or DAP2 (749 amino acids; 72 kDa; Palli *et al.* 1998), indicating they constitute a different class of hemolymph proteins. Therefore, we propose that the *O. furnacalis* DAP gene and homologous *O. nubilalis* genomic regions may encode ommochrome-binding proteins (OBPs).

A PredictProtein search of the ProSite database indicated the presence of two N-glycosylation sites, NITE and NKTK, from both *O. furnacalis* and *O. nubilalis obp* genes (Rost 1996; Fig. 1). A mannose attachment was found on the *M. sexta* OBP (Martel and Law 1991), and a single corresponding N-glycosylation site found by Yepiz-Plascencia *et al.* (1993). These predictions indicate the putative *Ostrinia obp*1 and *obp*2 gene products are also glycosylated. Additionally, a ProDom search (http://protein.toulouse.inra.fr/prodom/current/html/home.php) co-classified the *Ostrinia* and *M. sexta* OBPs within peptide Family PD125063 (Zdobnov and Apweiler 2001). All members of this protein family share a conserved glycosyl hydrolase family 10 active site that is present in *O. furnacalis* OBP residues 96 to 106 and *O. nubilalis* homologs (Fig. 1). Glycosyl hydrolases degrade cellulose and xylans, but the presence of this activity has not been previously described for OBPs. The function of this active site is unknown and it may be falsely identified by the Protein database. Nevertheless, the conserved domain provides evidence of co-ancestry between OBP encoding genes. Phylogenetic and expression level evidence for *O. nubilalis obp*1 and *obp*2 ommochrome binding protein classification also is shown (see below).

Gene homology and orthology

Phylogenetic methods can predict relationships and genealogical history among genes or alleles, and was used to explore common ancestry between lepidopteran *obp* genes. The parsimony-based phylogeny incorporated an alignment of 516 nucleotides shared by *O. furnacalis* DAP (OBP1) coding sequence (GenBank accession no. AF169311), *M. sexta* (Yepiz-Plascencia *et al.* 1993; GenBank accession no. L00975), *O. nubilalis obp*1 and *obp*2 allele fragments (Fig. 1), and *B. mori* expressed sequence tags fbVm022, ovS318H0, and ovS10G0. The resultant phylogeny showed that *Ostrinia obp* genes are nested within a clade with the *M. sexta* gene, indicating derivation from a single ancestral gene and common ancestry of contemporary genes. The tree also indicted ≥ 950 of 1000 bootstrap pseudoreplicates supported presence of a node between *O. nubilalis obp*1 and 2 alleles (Fig. 2). Lower interspecific compared to intraspecific similarity between On*obp*1 and On*obp*2 sequences and the phylogeny (Fig. 2) suggested duplication of On*obp*1 and On*obp*2 from a common ancestral gene. Gene duplications are maintained by gain of function or division of function (Force *et al.* 1999; Lynch and Force 2000), otherwise gene duplicates may accumulate mutations (“decay”) resulting in reduced or lost function and pseudogene formation.

Expression analysis

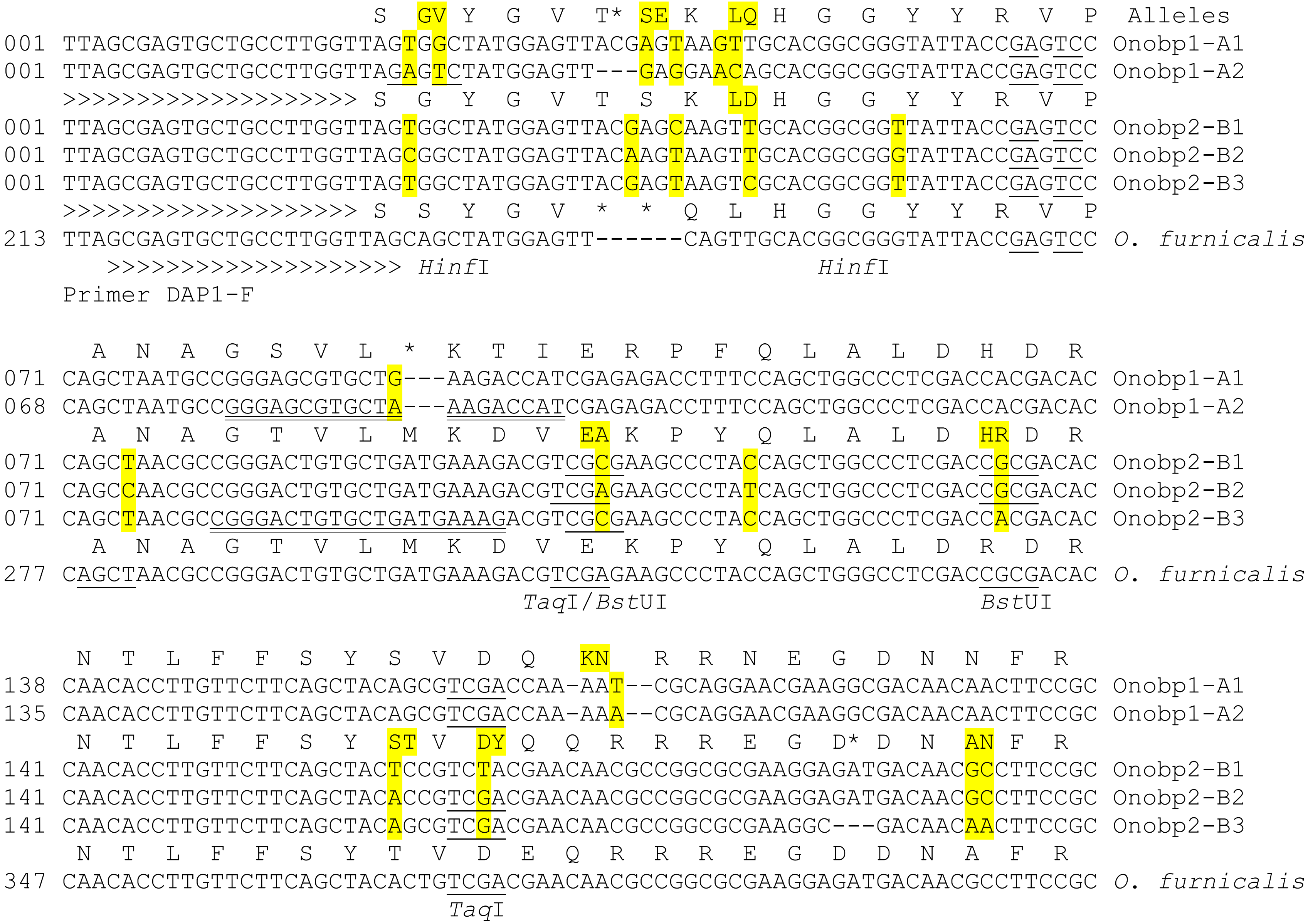
Semi-quantitative RT-PCR showed that transcription of On*obp*1 and On*obp*2 is differentially regulated in *O. nubilalis* fat body, and duplicate genes may be maintained in the genome due to their having different functions. The ommochrome-binding protein gene expressed in fat body of 5th instar *M. sexta* larvae (Yepiz-Plascencia *et al.* 1993) corresponded to a peptide that increased in concentration in hemolymph during late instars (Martel and Law 1991; 1992). An OBP protein was also purified from 5th instar *A. yamamai* hemolymph (Saito *et al.* 1998). Two co-expressed *obp*-like genes have not previously been described in Lepidoptera, except for the evidence presented here for larval *O. nubilalis* fat body. The tissue specificity of *O. nubilalis obp*1 and *obp*2 provides further evidence for correct classification of these genes as coding for ommochrome-binding proteins.

Expression patterns of two highly similar *O. nubilalis* genes (On*obp*1 and On*obp*2) were characterized by locus-specific RT-PCR. Results indicated On*obp*1 and 2 transcripts are present in total RNA preparations from whole larvae and dissected fat body tissue, but not in midgut or head tissues of 5th instar larvae (Fig. 3 A). Lack of *obp*1 and *obp*2 RT-PCR products suggests transcripts are not present or below the level of sensitivity in head and midgut tissues. Amplification of β-actin (b-actin) from total RNA obtained from each tissue indicated template integrity differences were not present between samples. Fat body expression of On*obp*1 and On*obp*2 transcripts was expected due to data from *M. sexta* (Yepiz-Plascencia *et al.* 1993) and further suggests correct classification as OBP-encoding genes.

The *O. nubilalis obp*1 gene has larval growth stage-specific expression. On*obp*1 transcript was detected by RT-RCR in total RNA from fat body of 5th instar larvae, whereas the transcript was not detected from 4th instar, 5th instar wandering, or 1 week old diapausing larval fat body using identical methods (Fig. 3 B). Similar larval instar-specific transcription was observed for a *M. sexta obp* but in 5th instar wandering larvae (Yepiz-Plascencia *et al.* 1993). Also, an *A. yamamai obp* peptide product was identified in 5th instar hemolymph (Saito *et al.* 1998). Data collected from *O. nubilalis*, *M. sexta*, and *A. yamamai* suggest an orthologous *obp* gene may be expressed in 5th and late 5th instar Lepidoptera.

In contrast to *obp*1, transcription of the *O. nubilalis obp*2 gene is not restricted to a particular developmental stage. The On*obp*2 transcript was amplified in fat body total RNA samples by RT-PCR from all larval growth stages tested, suggesting constitutive fat body expression (Fig. 3 B). The *obp*2 transcript level in the fat body may vary between larval stages. It increased as larvae moved from 4th instar to 5th instar wandering larvae, and remained high among 1 week old diapausing larvae. Assuming primers (DAP1-R, OBP1-F and OBP2-F) anneal with equal efficiency, the level of On*obp*2 transcription appears greater than On*obp*1. Higher overall OBP2 expression may result, although eukaryotic transcriptional and translational efficiency are not always correlated. Transcription of On*obp*2 across larval stages differed from the stage-specific expression of On*obp*1 (Fig. 3 B) and *M. sexta obp* transcripts (Yepiz-Plascencia *et al.* 1993). Differential expression of On*obp*1 and 2 among larval stages might suggest *O. nubilalis* paralogs might differ in developmental stage-specific enhancer elements (Ayer and Benyajati 1992).

Figure 1. *Ostrinia nubilalis* ommochrome binding protein gene 1 (On*obp*1; GenBank accession nos. AY819651 and AY819652) and 2 (On*obp*2; GenBank accession nos. AY819653, AY819654, and AY819655) alignments with *O. furnacalis* gene DAP (GenBank accession AF169311). The 350 nt sequence On*obp*2-B4 (GenBank accession AY862870) is from pedigree 10b male parent. A partial signal sequence is double underlined, a conserved glycosyl hydrolases family 10 active site is enclosed in a box, and an N-glycosylation site overwritten with an asterisk (*). Polymorphic sites are highlighted. Primer binding regions are underlined by arrows indicating directions, restriction endonuclease sites underlined, and location of gene specific primer double underlined.



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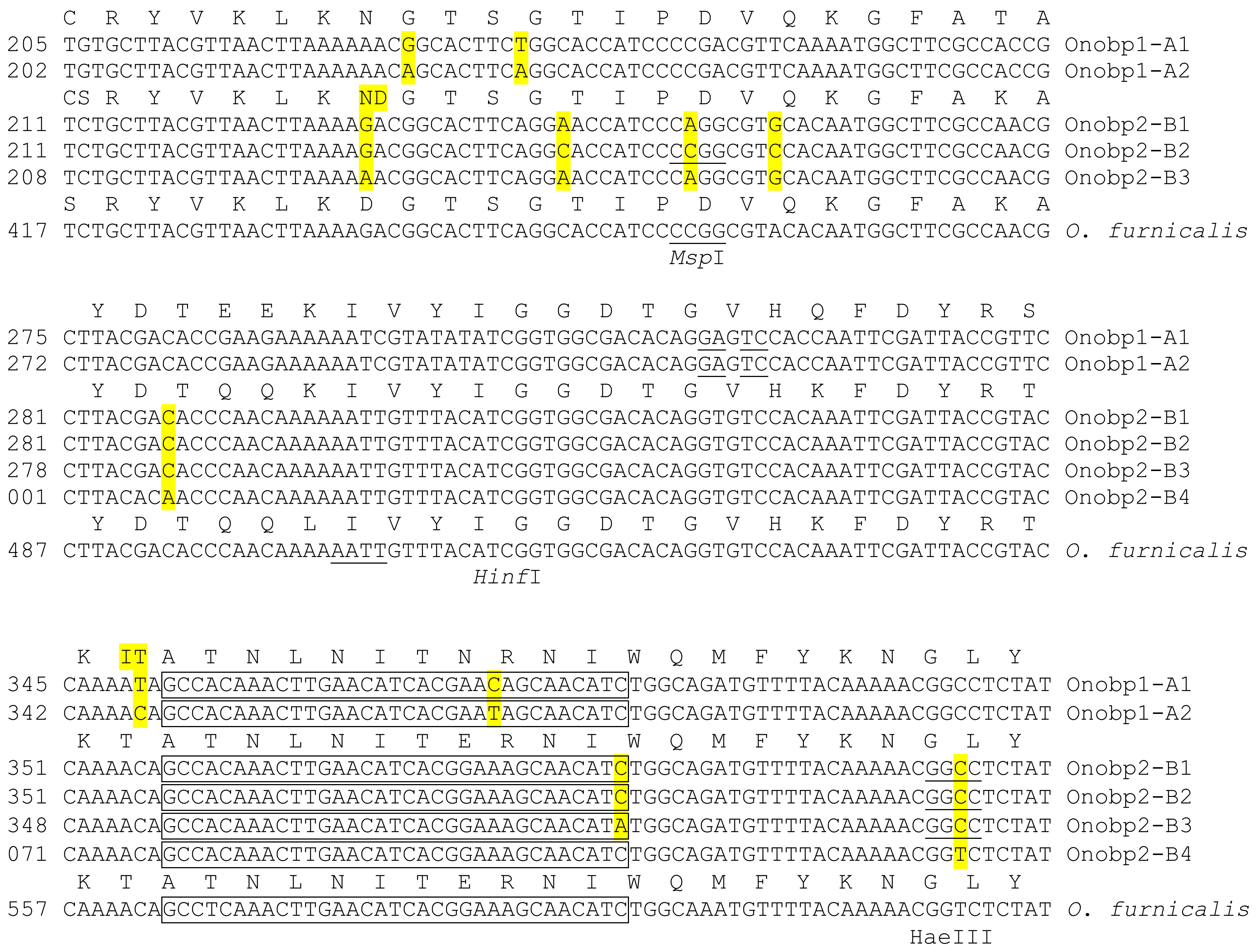
                         S  GV Y  G  V  T* SE K  LQ H  G  G  Y  Y  R  V  P  Alleles
001 TTAGCGAGTGCTGCCTTGGTTAGTGGCTATGGAGTTACGAGTAAGTTGCACGGCGGGTATTACCGAGTCC Onobp1-A1
001 TTAGCGAGTGCTGCCTTGGTTAGAGTCTATGGAGTT---GAGGAACAGCACGGCGGGTATTACCGAGTCC Onobp1-A2
    >>>>>>>>>>>>>>>>>>>> S  G  Y  G  V  T  S  K  LD H  G  G  Y  Y  R  V  P
001 TTAGCGAGTGCTGCCTTGGTTAGTGGCTATGGAGTTACGAGCAAGTTGCACGGCGGTTATTACCGAGTCC Onobp2-B1
001 TTAGCGAGTGCTGCCTTGGTTAGCGGCTATGGAGTTACAAGTAAGTTGCACGGCGGGTATTACCGAGTCC Onobp2-B2
001 TTAGCGAGTGCTGCCTTGGTTAGTGGCTATGGAGTTACGAGTAAGTCGCACGGCGGTTATTACCGAGTCC Onobp2-B3
    >>>>>>>>>>>>>>>>>>>> S  S  Y  G  V  *  *  Q  L  H  G  G  Y  Y  R  V  P
213 TTAGCGAGTGCTGCCTTGGTTAGCAGCTATGGAGTT------CAGTTGCACGGCGGGTATTACCGAGTCC O. furnicalis
       >>>>>>>>>>>>>>>>>>>> HinfI                      HinfI
    Primer DAP1-F

      A  N  A  G  S  V  L  *  K  T  I  E  R  P  F  Q  L  A  L  D  H  D  R
071 CAGCTAATGCCGGGAGCGTGCTG---AAGACCATCGAGAGACCTTTCCAGCTGGCCCTCGACCACGACAC Onobp1-A1
068 CAGCTAATGCCGGGAGCGTGCTA---AAGACCATCGAGAGACCTTTCCAGCTGGCCCTCGACCACGACAC Onobp1-A2
      A  N  A  G  T  V  L  M  K  D  V  EA K  P  Y  Q  L  A  L  D  HR D  R
071 CAGCTAACGCCGGGACTGTGCTGATGAAAGACGTCGCGAAGCCCTACCAGCTGGCCCTCGACCGCGACAC Onobp2-B1
071 CAGCCAACGCCGGGACTGTGCTGATGAAAGACGTCGAGAAGCCCTATCAGCTGGCCCTCGACCGCGACAC Onobp2-B2
071 CAGCTAACGCCGGGACTGTGCTGATGAAAGACGTCGCGAAGCCCTACCAGCTGGCCCTCGACCACGACAC Onobp2-B3
      A  N  A  G  T  V  L  M  K  D  V  E  K  P  Y  Q  L  A  L  D  R  D  R
277 CAGCTAACGCCGGGACTGTGCTGATGAAAGACGTCGAGAAGCCCTACCAGCTGGGCCTCGACCGCGACAC O. furnicalis
                                   TaqI/BstUI                     BstUI

     N  T  L  F  F  S  Y  S  V  D  Q   KN   R  R  N  E  G  D  N  N  F  R
138 CAACACCTTGTTCTTCAGCTACAGCGTCGACCAA-AAT--CGCAGGAACGAAGGCGACAACAACTTCCGC Onobp1-A1
135 CAACACCTTGTTCTTCAGCTACAGCGTCGACCAA-AAA--CGCAGGAACGAAGGCGACAACAACTTCCGC Onobp1-A2
     N  T  L  F  F  S  Y  ST V  DY Q  Q  R  R  R  E  G  D* D  N  AN F  R
141 CAACACCTTGTTCTTCAGCTACTCCGTCTACGAACAACGCCGGCGCGAAGGAGATGACAACGCCTTCCGC Onobp2-B1
141 CAACACCTTGTTCTTCAGCTACACCGTCGACGAACAACGCCGGCGCGAAGGAGATGACAACGCCTTCCGC Onobp2-B2
141 CAACACCTTGTTCTTCAGCTACAGCGTCGACGAACAACGCCGGCGCGAAGGC---GACAACAACTTCCGC Onobp2-B3
     N  T  L  F  F  S  Y  T  V  D  E  Q  R  R  R  E  G  D  D  N  A  F  R
347 CAACACCTTGTTCTTCAGCTACACTGTCGACGAACAACGCCGGCGCGAAGGAGATGACAACGCCTTCCGC O. furnicalis
                              TaqI
```

Figure 1. (Continued)



```
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202 TGTGCTTACGTTAACTTAAAAAACAGCACTTCAGGCACCATCCCCGACGTTCAAAATGGCTTCGCCACCG Onobp1-A2
    CS R  Y  V  K  L  K  ND G  T  S  G  T  I  P  D  V  Q  K  G  F  A  K  A
211 TCTGCTTACGTTAACTTAAAAGACGGCACTTCAGGAACCATCCCAGGCGTGCACAATGGCTTCGCCAACG Onobp2-B1
211 TCTGCTTACGTTAACTTAAAAGACGGCACTTCAGGCACCATCCCCGGCGTCCACAATGGCTTCGCCAACG Onobp2-B2
208 TCTGCTTACGTTAACTTAAAAAACGGCACTTCAGGAACCATCCCAGGCGTGCACAATGGCTTCGCCAACG Onobp2-B3
    S  R  Y  V  K  L  K  D  G  T  S  G  T  I  P  D  V  Q  K  G  F  A  K  A
417 TCTGCTTACGTTAACTTAAAAGACGGCACTTCAGGCACCATCCCCGGCGTACACAATGGCTTCGCCAACG O. furnicalis
                                                 MspI

     Y  D  T  E  E  K  I  V  Y  I  G  G  D  T  G  V  H  Q  F  D  Y  R  S
275 CTTACGACACCGAAGAAAAAATCGTATATATCGGTGGCGACACAGGAGTCCACCAATTCGATTACCGTTC Onobp1-A1
272 CTTACGACACCGAAGAAAAAATCGTATATATCGGTGGCGACACAGGAGTCCACCAATTCGATTACCGTTC Onobp1-A2
     Y  D  T  Q  Q  K  I  V  Y  I  G  G  D  T  G  V  H  K  F  D  Y  R  T
281 CTTACGACACCCAACAAAAAATTGTTTACATCGGTGGCGACACAGGTGTCCACAAATTCGATTACCGTAC Onobp2-B1
281 CTTACGACACCCAACAAAAAATTGTTTACATCGGTGGCGACACAGGTGTCCACAAATTCGATTACCGTAC Onobp2-B2
278 CTTACGACACCCAACAAAAAATTGTTTACATCGGTGGCGACACAGGTGTCCACAAATTCGATTACCGTAC Onobp2-B3
001 CTTACACAACCCAACAAAAAATTGTTTACATCGGTGGCGACACAGGTGTCCACAAATTCGATTACCGTAC Onobp2-B4
     Y  D  T  Q  Q  L  I  V  Y  I  G  G  D  T  G  V  H  K  F  D  Y  R  T
487 CTTACGACACCCAACAAAAAATTGTTTACATCGGTGGCGACACAGGTGTCCACAAATTCGATTACCGTAC O. furnicalis
                           HinfI

   K  IT A  T  N  L  N  I  T  N  R  N  I  W  Q  M  F  Y  K  N  G  L  Y
345 CAAAATAGCCACAAACTTGAACATCACGAACAGCAACATCTGGCAGATGTTTTACAAAAACGGCCTCTAT Onobp1-A1
342 CAAAACAGCCACAAACTTGAACATCACGAATAGCAACATCTGGCAGATGTTTTACAAAAACGGCCTCTAT Onobp1-A2
   K  T  A  T  N  L  N  I  T  E  R  N  I  W  Q  M  F  Y  K  N  G  L  Y
351 CAAAACAGCCACAAACTTGAACATCACGGAAAGCAACATCTGGCAGATGTTTTACAAAAACGGCCTCTAT Onobp2-B1
351 CAAAACAGCCACAAACTTGAACATCACGGAAAGCAACATCTGGCAGATGTTTTACAAAAACGGCCTCTAT Onobp2-B2
348 CAAAACAGCCACAAACTTGAACATCACGGAAAGCAACATATGGCAGATGTTTTACAAAAACGGCCTCTAT Onobp2-B3
071 CAAAACAGCCACAAACTTGAACATCACGGAAAGCAACATCTGGCAGATGTTTTACAAAAACGGTCTCTAT Onobp2-B4
   K  T  A  T  N  L  N  I  T  E  R  N  I  W  Q  M  F  Y  K  N  G  L  Y
557 CAAAACAGCCTCAAACTTGAACATCACGGAAAGCAACATCTGGCAAATGTTTTACAAAAACGGTCTCTAT O. furnicalis
                                                              HaeIII
```

Figure 1. (Continued)

```
    F  T  T  Y  P  D  Q  K  A  F  I  Y  K  N  G  K  V  N  L  V  P  E  L  Q
415 TTTACCACTTATCCAGACCAGAAAGCGTTCATTTACAAAAATGGCAAAGTTAACCTCGTGCCGGAACTGC Onobp1-A1
412 TTCACCACTTATCCAGACCAGAAAGCGTTCATTTACAAAAATGGCAAAGTTAACCTCGTGCCGGAACTGC Onobp1-A2
    F  T  T  Y  P  D  Q  K  A  F  V  Y  K  N  DG S  L  A  L  V  P  E  L  M
421 TTCACCACTTATCCTGACCAGAAAGCGTTCGTTTACAAAAATGGCAGGCTTAGACTCGTGCCGGAACTGA Onobp2-B1
421 TTCACCACCTATCCTGACCAGAAAGCGTTCGTATACAAAAATGGCAGGCTTAGACTCGTGCCGGAACTGA Onobp2-B2
418 TTCACCACCTATCCTGACCAGAAAGCGTTCGTTTACAAAAATGACAGGCTTAGACTCGTGCCGGAACTGA Onobp2-B3
141 TTCACCACTTATCCTGACCAGAAAGCGTTCGTTTACAAAAATGACAGGCTTAGACTCGTGCCGGAACTGA Onobp2-B4
    F  T  T  Y  P  D  Q  K  A  F  V  Y  K  N  D  S  L  A  L  V  P  E  L  M
627 TTCACCACTTATCCTGACCAGAAAGCGTTCGTTTACAAAAATGACAGGCTTAGACTCGTGCCGGAACTGA O. furnicalis
                                                 HinfI

     D  LS  K  A  M  M  V  A  V  D  KN DT D  S  V  V  Y  S  LP H  G  V  L
485 AAGACTTAAAGGCGATGATGGTGGCCGTGGACAAGGATGATAGTGTAGTTTACTCTTTGCATGGGGTCCT Onobp1-A1
482 AAGACTCAAAGGCGATGATGGTGGCCGTGGACAACACTGATAGTGTAGTCTACTCTCCGCATGGGGTCCT Onobp1-A2
      D  V  K  A  T  L  V  A  L  D  T  D  D  PS I  V  Y  S  L  D  G  D  L
491 TGGACGTAAAGGCAACGCTGGTGGCCCTGGAAAAGGATGATAGTATAGTCTACTCTTTGGATGGGGACCT Onobp2-B1
491 TGGACGTAAAGGCAACGCTGGTGGCCCTGGAAAAGGATGATAGTATAGTCTACTCTTTGGATGGGGACCT Onobp2-B2
488 TGGACGTAAAGGCAACGCTGGTGGCCCTGGAAAAGGATGATAGTATAGTCTACTCTTTGGATGGGGACCT Onobp2-B3
211 TGGACGTAAAGGCAACGCTGGTAGCCCTGGAAAAGGATGATAGTATAGTTTACTCTTTGGATGGGGACCT Onobp2-B4
      D  V  K  A  T  L  V  A  L  D  K  G  D  S  I  V  Y  S  L  D  G  D  L
697 TGGACGTTAAGGCAACGCTGGTGGCCCTGGAAAAGGGTGATAGTATAGTTTACTCTTTGGATGGGGACCT O. furnicalis
    HinfI/MseI           HaeIII

     R  R  A  S  E  G  R  I  DY E  L  G  S  Y  N  V  N  G  F  N  T  D  V
555 GAGGCGGGCTTCTGAGGGGCGGATCGACGAGCTGGGCTCGTACAATGTGAATGGTTTCAACACTGACGTC Onobp1-A1
552 GAGGCGGGCTTCTGAGGGGCGTATCTACGAGCTGGGCTCGTACAATGTGAATGGTTTCAACACTGACGTC Onobp1-A2
     T  R  A  S  E  G  R  I  Y  D  L  G  S  Y  I  V  I  W  F  N  T  D  V
561 GAGGCGGACTTCTGAGGGGCGGATCTACGAGCTGGGCTCGTACATTGTAATATGGTTCAACACTGACGTC Onobp2-B1
561 GAGGCGGACTTCTGAGGGGCGGATCTACGAGCTGGGCTCGTACATTGTAATATGGTTCAACACTGACGTC Onobp2-B2
558 GAGGCGGACTTCTGAGGGGCGGATCTACGAACTGGGCTCGTACATAGTAATATGGTTCAACACTGACGTC Onobp2-B3
281 GAGGCGGACTTCGGAGGGGCGGGTCTACGAGCTGGGCTCGTACAATGTGAATGGTTTCAACACTGACGTC Onobp2-B4
     R  R  T  S  E  G  R  V  Y  E  L  G  S  Y  N  V  N  G  F  N  T  D  V
767 GAGGCGGACTTCGGAGGGGCGGGTCTACGAGCTGGGCTCGTACAATGTGAATGGTTTCAACACTGACGTC O. furnicalis
                          NdeII
```

Figure 1. (Continued)

```
    N  G  D L  Y  F  S  T  S  D  A  I  Y  Q  V  D  G  N  T  V  Q  K  F  A
625 AACGGTGACTTGTACTTCTCGACATCGGATGCTATTTACCAGGTTAACGGGAACACTGTGCAGAAGTTCG Onobp1-A1
622 AACGGTGACTTGTACTTCTCGACATCGGATGCTATTTACCAGGTTAACGGGAACACTGTGCAGAAGTTCG Onobp1-A2
    N  G  D  L  Y  F  S  T  F  D  A  I  Y  K  V  EN G  N  A  V  R  K  F  A
631 AACGGTGACTTGTACTTCTCGACTTTTGATGCTATTTACAAGGTTGAAGGGAACGCTGTGCGGAAGTTCG Onobp2-B1
631 AACGGTGACTTGTACTTCTCGACTTTTGATGCTATTTACAAGGTTGAAGGGAACGCTGTGCGGAAGTTCG Onobp2-B2
628 AACGGTGACTTGTACTTCTCAACATTGGATGCTATTTACCAGGTTAACGGGAACGCTGTGCGGAAGTTCG Onobp2-B3
    N  G  D  L  Y  F  S  T  S  D  A  I  Y  Q  V  D  G  N  T  V  Q  K  F  A
837 AACGGTGACTTGTACTTCTCGACATCGGATGCTATTTACCAGGTTGACGGGAACACTGTGCAGAAGTTCG O. furnicalis
                      TaqI                      MseI

      A  F  P  D  I  Y  G  F  A  F  E  G  D  K  Q  I  I  Y  G  T  E
695 CTGCGTTTCCCGACATATATGGGTTTGCTTTCGAGGGGGACAAGCAGATCATCTACGGAACAGAGAACAG Onobp1-A1
692 CTGCGTTTCCCGACATATATGGGTTTGCTTTCGAGGGGGACAAGCAGATCATCTACGGAACAGAGAACAG Onobp1-A2
      A  F  P  D  I  Y  G  F  A  F  E  G  D  K  Q  I  I  Y  G  T  E
701 CTGCATTTCCCGACATATATGGGTTTGCTTTCGAGGGGGACAAGCAGATCATCTACGGAACAGAGAACAG Onobp2-B1
701 CTGCATTTCCCGACATATATGGGTTTGCTTTCGAGGGGGACAAGCAGATCGTCTACGGAACAGAGAACAG Onobp2-B2
698 CTGCGTTTCCCGACATCTATGGGTTTGCTTTCGAGGGGGACAAGCAGATCATCTACGGAACAGAGAACAG Onobp2-B3
      A  F  P  D  I  Y  G  F  A  F  E  G  D  K  Q  I  I  Y  G  T  E  N  S
907 CTGCGTTTCCCGACATCTATGGGTTTGCTTTCGAGGGGGACAAGCAGATCATCTACGGAACAGAGAACAG O. furnicalis
                                                                     <<<<<

765 TGTGATGCGCCTGAC Onobp1-A1 (GenBank accession no. AY819651)
762 TGTGATGCGCCTGAC Onobp1-A2 (GenBank accession no. AY819652)
771 TGTGATGCGCCTGAC Onobp2-B1 (GenBank accession no. AY819653)
771 TGTGATGCGCCTGAC Onobp2-B2 (GenBank accession no. AY819654)
768 TGTGATGCGCCTGAC Onobp2-B3 (GenBank accession no. AY819655)
                    Onobp2-B4 (GenBank accession no. AY862870)
     V  M  R  L  T
977 TGTGATGCGCCTGAC O. furnicalis(GenBank accession no. AF169311)
    <<<<<<<<<<<<<<<
      Primer DAP1-R
```

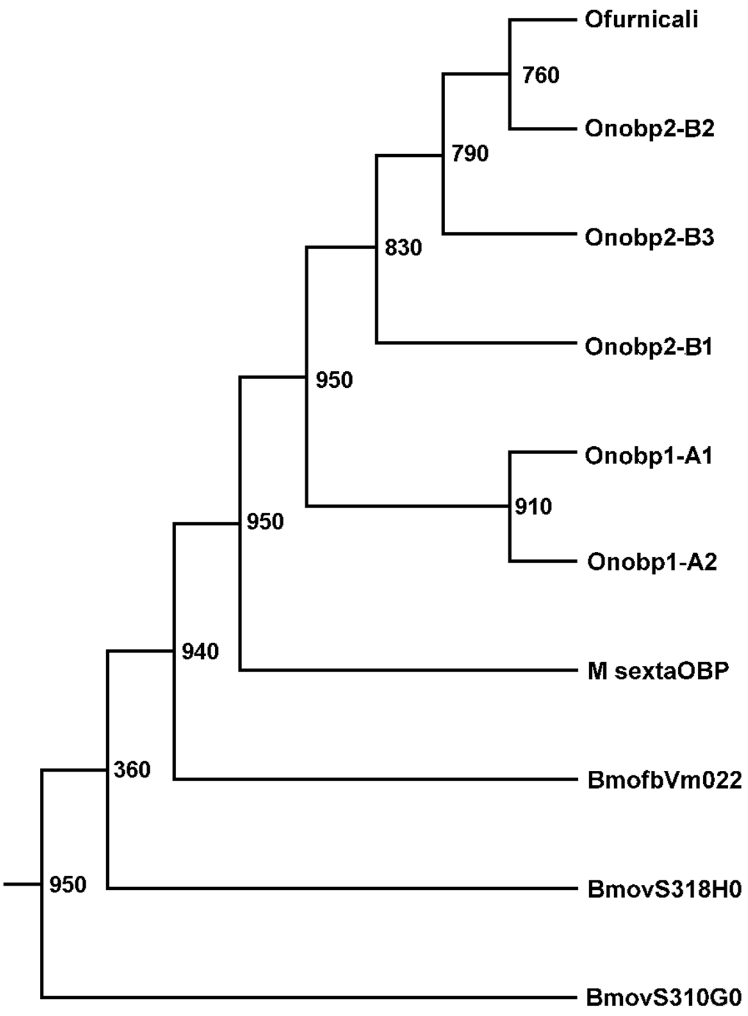

Figure 2. Ommochrome-binding protein gene (obp) phylogeny generated from a 516 nt consensus alignment from nucleotide positions 246 and 762 of the O. *furnacalis* diapause associated polypeptide (DAP; GenBank accession no. AF169311). Other OBP gene sequences include *O. nubilalis ob*p1 alleles Onobp2-A1 and -A2, and obp2 alleles Onobp2-A1, -A2, and -A3 (See Fig. 1), *M. sexta* (M Sexta OBP; GenBank accession no. L00975), and *Bombyx mori* (Bm) expressed sequence tag sequences from fat body (fbVm022) and ovarian tissue (ovS318H0, and ovS10G0).

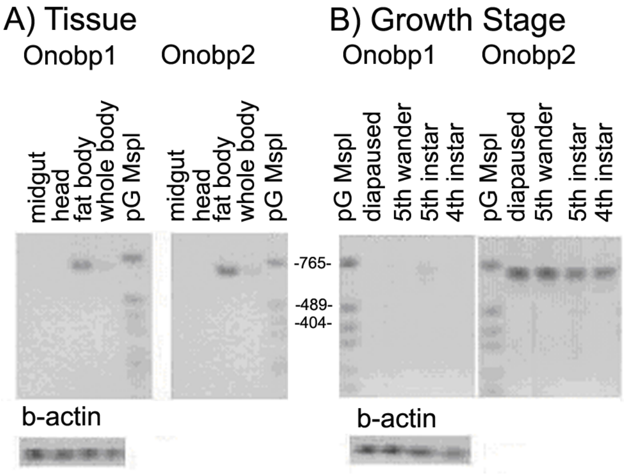

Figure 3. Reverse transcriptase (RT)-PCR detection of *Ostrinia nubilalis* ommochrome binding protein 2 (*obp*2) and 1 (*obp*1) gene transcripts in fat body tissue from 4th and 5th instar larvae, 5th instar wandering larvae, and 1 wk diapausing larvae. RT-PCR of the β-actin (b-actin) gene was used as a control for integrity of total RNA samples from each tissue.

Ostrinia nubilalis ommochrome binding protein polymorphism

Four peptide sequence conflicts were identified within the first 31 residues of *M. sexta* OBP N-terminal sequence by comparing evidence from Martel and Law (1991) and Yepez-Plascencia *et al.* (1993). These data suggest variability exists even within the same species, and may contribute to low peptide-level similarity and identity between species. Two unique sequence types with similarity to the *O. furnacalis* DAP gene (reclassified as OBP1) were isolated from *O. nubilalis* by PCR, (GenBank accession nos. AY819651 to AY819655 and AY862870). No introns were predicted within either gene fragment. Intraspecific alignment of 775 to 778 bp On*obp*1 (alleles On*obp*1-A1, and -A2) and 782 to 785 bp On*obp*2 gene fragments (alleles On*obp*2-B1, -B2, and -B3) identified 21 and 23 single nucleotide polymorphisms, respectively (Fig. 1). On*obp*1 single nucleotide polymorphisms within *Hinf*I or *Tsp*509I, and On*obp*2 mutations within *Hae*III, *Mse*I or *Taq*I restriction endonuclease cleavage sites were observed (Fig. 1; Table 1).

The difference of three nucleotides was due to a Thr deletion near the N-terminus of On*obp*1, and an Asp residue deletion among On*obp*2 sequences. DNA sequence data indicated 97.0% and 97.3 to 98.3% similarity between On*obp*1 and 2 alleles, respectively. Lower sequence similarity was observed between On*obp*1 and 2 sequences (88.3 to 90.1%). The On*obp*2-B4 sequencing fragment (GenBank accession no. AY862870) was not included in this analysis, but used to identify polymorphic nucleotides within pedigrees. Lower similarity between On*obp*1 and On*obp*2 might suggest sequences are from separate loci. Sequence differentiation can occur between alleles at the same locus, thus similarity comparisons between On*obp*1 and On*obp*2 do not prove they are unique genes with different genome positions. Fidelity of allelic inheritance was confirmed by pedigree analysis.

Mendelian inheritance

Two sets of divergent yet similar genome sequences (On*obp*1 and On*obp*2) isolated from *O. nubilalis* by PCR constitute separate loci. Gene specific primer fidelity was inferred by calculating degree of mismatch and estimated melting temperature. The On*obp*1 specific OnOBP1-F primer showed 25% mismatch to the homologous region of On*obp*2 template sequence with an estimated annealing temperature ≅ 48 °C. The On*obp*2 locus specific OnOBP2-F primer had 30% mismatch with homologous On*obp*1 template with an estimated annealing temperature of ≅ 46 °C. Template (gene or locus) specificity of primers was shown empirically by failure of OnOBP1-F to prime PCR that used a cloned On*obp*2 gene fragment as template, and OnOBP2-F primer to prime PCR that used a cloned On*obp*1 gene fragment as template (58° C annealing;

Table 1. Mendelian inheritance of *obp*1 (locus A) and *obp*2 (locus B) alleles in *O. nubilalis* pedigrees Ped10b and Ped24a. Observed genotypic ration among F_2 offspring was compared to Mendelian expected F_2 ratio based on parental genotypes using PCR-RFLP. Allele names correspond to sequences in Fig. 1. No significant allele frequency departures from expected was observed by chi-square (χ^2) tests.

Pedigree	Parental genotype	Observed PCR-RFLP genotype ratios among F_2 progeny	Expected F_2 ratio	chi^2	*p* -value (df)
Ped10b	*obp* 1:	*obp* 1:			
*Hinf*I	♂A_1A_2	A_1A_1 (16/32): A_1A_2 (10/32): A_2A_2 (6/32)	9:6:1	4.81	0.091 (2)
	♀A_1A_1				
	obp 2:	*obp* 2:			
Hae III	♂B_4B_4	B_4B_4(21/39):B_4B_1(18/39):B_1B_1(0/39)	9:6:1	3.26	0.196 (2)
	♀B_1B_4				
Ped24a	*obp* 1: ♂A_2A_2	*obp* 1:			
*Hinf*I	♀A_1A_1	A_1A_1(9/33): A_1A_2(18/33): A_2A_2(6/33)	1:02:01	0.82	0.664 (2)
	obp 2:	*obp* 2:			
	♂B_4B_4	No variation detected			
	♀B_4B_4				

data not shown).

Observed F_2 ratios indicated On*obp*1 *Hinf*I PCR-RFLP or On*obp*2 *Hae*III PCR-RFLP defined alleles did not deviate significantly from Mendelian expectation (Table 1). In combination with sequence specificity of forward primers, phylogeny, differential expression, Mendelian segregation of heterozygous allele states in On*obp*1 and On*obp*2 assays indicate PCR products are derived from unique loci, and show that null alleles were not present (Pemberton *et al*. 1995).

Concluding Remarks

Two *O. nubilalis* ommochrome binding protein paralogs, On*obp*1 and On*obp*2, are transcribed by fat body cells, suggesting retention of a shared fat body specific promoter elements (Ayre and Benyajati 1992). Contemporary status of gene duplication may be that, 1) both copies may retain similar function, 2) copies may have divergent function, or 3) one copy may have decayed or be in process of degrading into a pseudogene. Divergence in duplicate gene function encompasses the gain and division of function of which the latter includes temporal or tissue-specific expression (Force *et al*. 1999; Lynch and Force 2000). Changes in duplicated genes are more likely to be in the patterning of expression as opposed to diversification of protein or enzyme function (Purugganan 1998; Wendel 2000), and may explain the large eukaryotic gene families (Hartl and Clarke 1997).

Differences in temporal expression of *O. nubilalis obp*1 and *obp*2 may constitute an example of division of function and change in duplicate gene function by differential expression. Fifth instar specific transcription of On*obp*1 may suggest a physiological requirement only prior to or during diapause induction (Fig. 3). Pre-diapause OBP1 expression may coincide with increased larval metabolic activities when energy storage demands are highest. Triggering On*obp*1 transcription may facilitate a greater detoxification capacity. Alternatively, On*obp*1 expression might be an evolutionary flicker as promoter region mutations accumulate suggesting On*obp*1 may be decaying into a pseudogene (Marshall *et al*. 1994; Nei *et al*. 1997). In contrast, constitutive expression of On*obp*2 may indicate a universal physiological demand for OBP2 function. Additional investigation into promoter region structure and function, and potential OBP1 and OBP2 function will be required to elucidate contemporary contribution to diapause physiology and maintenance of extended periods of diapause in univoltine ecotypes.

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