## CHAPTER 3

# Results

#### 3.1 Molecular characterization of OfDH-PBAN cDNA

# 3.1.1 Cloning of OfDH-PBAN cDNA

Using the degenerated primers OfDPF and OfDPR (Fig. 3.1), an amplified DNA fragment of the expected 177 bp size was obtained. After cloning and sequencing, the deduced amino acid sequence shows 57-63% identity to other known DH-PBAN cDNAs. Two specific primers, OfDP-SPF for the 3'-RACE and OfDP-SPR for the 5'-RACE (Fig. 3.1), were synthesized based on the sequence of the 402 bp fragment. After amplification and cloning, a 493 bp fragment from 5'-RACE and a 533 bp fragment from 3'-RACE were obtained.

The full-length cDNA (787 bp) contains a 5' untranslated region of 27 nucleotides, and the open reading frame (ORF) represents 597 nucleotides encoding a 199-amino acid long polypeptide. The ORF is terminated by a TAA stop codon that is followed by a 160-nucleotide long 3' untranslated region. A consensus polyadenylation signal (ATTAAA) was found 9 bp upstream of the polyA tail. A hydrophobic sequence from M<sup>1</sup> to V<sup>23</sup> serves as a signal peptide (Von Heijne, 1985). There are six potential endoproteolytic cleavage sites at G<sup>47</sup>-K<sup>48</sup>-R<sup>49</sup>, K<sup>95</sup>-K<sup>96</sup>, G<sup>104</sup>-R<sup>105</sup>, G<sup>126</sup>-R<sup>127</sup>-R<sup>128</sup>, G<sup>166</sup>-R<sup>167</sup> and G<sup>176</sup>-R<sup>177</sup> (Fig. 5). Consequently, the cDNA is believed to encode the precursor polyproteins for DH at D<sup>24</sup>-L<sup>46</sup>,  $\alpha$ -SGNP at V<sup>97</sup>-L<sup>103</sup>,  $\beta$ -SNGP at S<sup>106</sup>-L<sup>125</sup>, PBAN at L<sup>129</sup>-L<sup>165</sup> and  $\gamma$ -SGNP at T<sup>168</sup>-L<sup>175</sup>, all of which share the same C-terminal motif FXPR/KL (Fig. 3.2A).

By homology search, the *OfDH-PBAN* amino acid sequence deduced from the other known *DH-PBANs* shows the following homology: 74% with *M. vitrata*, 64% with *Antheraea pernyi*, 63% with *H. armigera*, 62% with *S. exigua*, 61% with *B. mori* and 54% with *Plutella xylostella* (Table 3.1). At the amino acid level, *OfDH* is 35-92%

identical to other known *DHs*, and *OfPBAN* is 35-60% identical to other known *PBANs*. *Of-\alpha-SGNP* is 86-100% identical to those from other species, whereas *Of-\beta-SGNP* and *Of-\gamma-SGNP* show 38-75% and 50-88% similarity with those from other species (Fig. 3.2B).

GAAC	AACA	TCCC	CTTA	ACCA	AATI	AAG	ATG M	TCT S	ATT I	TTT F	AAC N	TTG L	AAA K	TTT F	GTA V	54 9
TTG L	TCT S	ATT I	TTC F	GCT A	TTG L	TTC F	TGT C	GGA G	TTT F	GCG A	ACG T	GCG A	GTT V	GAT D	GAT D	102 25
TTG L	AAG K	GAT D	GAA E	GCA A	GAC D	CGC R	GGG G	GCC A	AGT S	GA T D	CGT R	GGA G	ACC T	CTT L	TGG W	150 41
		OfD:	PF					DH								
TTT	GGA	CCT	CGG	TTG	GGC	AAA	CGC	TCC	CTA	AGG	ATC	TCT	AAT	GAC	GAC	198
F	G	D	R	Τ.	G	K	P	DP-3	T.	R	т	S	N	D	D	57
1	0	1	I	1	0	K	Of	DP-S	PF	IX.	1	5	IN	D	D	57
AAT	AGG	CAA	ACC	TTC	CTT	AGA	CTA	TTG	GAG	GCT	GĆA	GAC	GCT	CTG	AAG	246
Ν	R	Q	Т	F	L	R	L	L	E	A	A	D	A	L	К	73
101111		1000		12.12			121-221-2			(	OfDH	PF		->		
TAC	TAC	TAC	GAC	CAG	CTA	CCT	TTC	TAT	GAG	AGT	CGA	GCT	GAT	GAC	CCT	294
Y	Y	Y	D	Q	L	P	F	Y	E	S	R	A	D	D	Р	89
CDD	ACT	CCC	CTTA	D CD	777	DIDPE	CTC	D.T.C.	<b>TTTC</b>	DCD	CCC	תתת	CTC	CCT	CCC	212
GAA	AC I T	R	V	ACA T	K	RAG	V	T	F	ACA T	P	K	L.	GGI	P	105
	1	I	•	1	K	K	<u> </u>	-	α.	-SGN	P	IX		0	K	100
AGC	ATG	GAT	GGC	TAC	TCC	GAC	AAA	CGG	ACG	TAT	GAG	AAC	GTA	GAG	TTC	390
S	М	D	G	Y	S	D	К	R	Т	Y	Е	Ν	V	Е	F	121
1			β-3	SGNP			-	(		OfD	HPR				-	
ACT	CCT	CGG	CTC	GGA	AGG	AGA	CTG	CCG	GAG	AAG	CTT	TCC	GTC	ACG	CCC	438
T	Р	R	L	G	R	R	L	Р	E	K	L	S	V	Т	Р	137
TCG	GAT	TCT	CAT	GAT	GCG	GTA	TAC	AGT	TTC	AAA	CCA	GAA	ATG M	AGT	GAA	486
5	D	5	п	D	A	V	P	BAN	Ľ	Л	P	Б	PI	5	Ľ	100
TTG	GAC	TCG	CGG	AAC	AAC	TAC	TTC	TCG	CCA	CGA	CTC	GGC	AGG	ACT	GTC	534
L	D	S	R	Ν	Ν	Y	F	S	Р	R	L	G	R	Т	V	169
8							_							_		
AAC	TTC	TCA	CCA	AGA	TTA	GGC	AGG	GAA	CTG	TCA	TAC	GAT	ATC	TAT	CCA	582
N	F	S	Р	R	L	G	R	E	L	S	Y	D	I	Y	Ρ	185
CDC	Ŷ	-SGN	P	ama	007	7	D.C.C.	700	770	mm.c	Taa		TOT		TOTO	6.01
GAG	AAG	ATA	AGG	CTG	GCA	AGA	AGC	ATT	AAC	TTG	ACC	AAA	ACA	TAA	TGAC	63L 100
Ľ	N	Т	R	Ц	A	Л	5	Т	IN	Ц	T	N	T			199
AACO	GAAT	TAAC	ATTAZ	AAAA	CCGT	ACTT	TAGT	ГААА	AGTA	GGTA'	ГТТТ	AAAC	GGAT	GACA	AGTG	694
TATO	CGCG	IGAA	CTTAC	GCAA'	TTTT	AAAT	AATG	AAAA	ATTT	ATAA	AAAC	AAGG	AAAA	TGAT	TCGG	757
CAAA	AAAA	AAAA	AAAA	AAAA	AAAA	AAA	AAA								40 MERIES (1957)	787

Figure 3.1 Nucleotide sequence and deduced amino acid sequence of the *OfDH-PBAN* cDNA. The suggested start (ATG) and stop (TAA) codons, and polyadenylation signal (ATTAAA) are shown in bold letters. The five presumptive peptides are underlined, and endoproteolytic cleavage sites are printed in bold. Arrows over the nucleotide sequences

represent the position of the different synthetic primers used in PCR. Degenerate primers are OfDPF (5'-TGG TTC GGH CCY AGR HTN GGS-3') and OfDPR (5'-GAA GAT BAC YTT YTT BGT HAC-3'). Specific primers for RACE and PCR are OfDP-SPF (5'-CTT AGA CTA TTG GAG GCT GCA-3') and OfDP-SPR (5'-GAT CCT TAG GGA GCG TTT GCC-3').



Species       DH       a-SGNP       p-SGNP       PBAN         0. fuscidentalis      VDDLKD-EADRGA-SDRGT-LMFGPRL       (%)       VIFTPKL       (%)	в											
0.fuscidentaliavDLLkD-EADRGA-SDRGT-LMTGPER(%)VIFTPKL(%)-SMDGYSD-KRYTENVEFTPRL(%)-LEKLSVTPSDSHDAVYSPKDEMSELDSR-NYTSPERL(%)M. vitrataLDDSKD-EADRGA-SDRGT-LMTGPER92VVFTPKL86SIGGVFQDKKYDNVEFIPRL70IPDALFVTPSDDDVYSFKDDSGEVDRR-TSYPNERL60S. d. riciniRNDVKD-ECORGAHSDRGA-LMTGPER79VIFTPKL100RANAVGE-KRYTENVEFIPRL50IEDEMPATPSDQEYPMYHDDPEQIDTR-TRYFSFRL10A. peknyiSNDIKD-ECORGAHSDRGA-LMTGPER75VIFTPKL86SIGDIYGE-KRYTENVEFIPRL50ISEDMPATPSDQEYPMYHDDPEQIDTR-TRYFSFLL16B. moriTDMQD-ESDRGAHSRGA-LMTGPER76VIFTPKL86SVAKPQTHESLEFIPRL50ISEDMPATPADQE-MYOPDPEMESR-TRYFSFLL16G. anastomosisINNDNTMCOGADRGAHSDRGG-LMTGPER76VIFTPKL86SKAVDDKSYENVEFTPRL67IADDPATPADQE-MYRDPPEQIDRS-TRYFSFLL17G. thyellinaSNDIXCDGQADRAHSDRGG-LMTGPER75VIFTPKL86SKAVDDKSYENVEFTPRL61IADDPATPADQE-MYRDPPEQIDSR-TKYFSFLL18G. thyellinaNNNDXCDGQADRAHSDRGG-LMTGPER71VIFTFKL100SISTYEEKLYDNVEFTPRL61IADDMATPADQE-MYRDPEQIDSR-TKYFSFLL14H. armigeraNNNDXCDGGAAGAHSDRGL-LMTGPER67VIFTKL100SISTYEEKLYDNVEFTPRL61ISDDMATPADQE-MYRQPEQIDSR-TKYFSFLL14H. assultaNNNDXCDGGAAGAHSDRGL-LMTGPER67VIFTKL100SISTYEEKLYDNVEFTPRL61ISDDMATPADQE-MYRQPEQIDSR-TKYFSFLL<		Species	DH		α-SGN	P	β-SGNP		PBAN		Y-SGNP	
MvirtataLDDSKD-EADRGA-SDRGT-LWFGFR92VVFTKL86SIGUFQDKKYDNVEFIFL47IPDALFVTPSDDDVYSFKPOSGEVDRR-TSYFNPL60S. c. riainiTMDVKD-EGDRGAHSDRGS-LWFGFR79VIFTKL100RASNAYQE-KRYTENVEFTFL70ILTEDMPATPTDQEMFDQDP2QIDTR-TRYFSFRL40A. sextaSNDIKD-EGDRGAHSDRGA-LWFGFR79VIFTEL66SIGDIYQE-KRYTENVEFTFL50SIEDMPATPSDQEYPMYHPDP2QIDTR-TRYFSFL40B. moriSNDIKD-EGDRGAHSDRGA-LWFGFR70VIFTEL60SIDDYGEKRYFYENFEFTEL50SIEDMPATPADQEMYQDP2EVMESR-TRYFSFL41B. madarinaTDMCD-ESDRGAHSERGA-LWFGFR70VIFTEL60SNAVDDKSYENVEFTEL60SIEDMPATPADQEMYQDP2EVMESR-TRYFSFL42C. anastomosisINNNITMOGGADRGAHSDRG-LWFGFR70VIFTEL60-SNAVDDKSYENVEFTEL61SIEDMPATPADQEMYRDP2QIDSR-TRYFSFL43A. ipsilonSNRDVCDGADRGAHSDRG-LWFGFR71VIFTEL100-SISTYEEKLYDNVEFTEL61SIEDMPATPADQEMYRDP2QIDSR-TRYFSFL44A. ipsilonNNNUKD-GAASGAHSDRG-LWFGFR61VIFTEL100SISTYEEKLYDNVEFTEL61SIEDMPATPADQEMYRDP2QIDSR-TRYFSFL45A. insigaraNNNUKD-GAASGAHSDRG-LWFGFR61VIFTEL100SISTYEEKLYDNVEFTEL61SIEDMPATPADQEMYRDP2QIDSR-TRYFSFL41H. armigaraNNNUKD-GAASGAHSDRG-LWFGFR61VIFTEL100SISTYEEKLYDNVEFTEL61SIEDMPATPADQEMYRDP2QIDSR-TRYFSFL41H. armigaraNN	о.	fuscidentalis	VDDLKD-EADRGA-SDRGT-LWFGPRL	(%)	VIFTPKL	(%)	-SMDGYSD-KRTYENVEFTPRL	(%)	-LPEKLSVTPSDSHDAVYSFKPEMSELDSR-NNYFSPRL	(%)	TVNFSPRL	(%
S. c. riciniTNDVKD-EGDRGAH3DRGS-LMFGPR79VI FITFKL100RANAYQE-KRTYENVEFTFRL70-ITEDMATPTDQE-ME-DQDPQQIDTR-TRYFSPRL12M. sextaSNDIKD-EGDRGAH3DRGA-LMFGPRL79VI FITFKL66SIGDIYQE-KRTYENVEFTFRL55-ISEDMATP3DQE-YMHPDPQQIDTR-TRYFSPRL13A. pernyiSNDIKD-EGDRGAH3ERGA-LMFGPRL75VI FITFKL66SNAKQTHSLEFIFRL50-ISEDMATP3DQE-MYQPDPEMESR-TRYFSPRL47B. moriTDMKO-ESDRGAH3ERGA-LMFGPRL78VI FITFKL86SNAKQTHSLEFIFRL44-ISEDMATATAQCE-MYQPDPEMESR-TRYFSPRL47C. anastomosisTNNDNTMCGGADGAH3DRGG-LMFGPRL71VI FIFKL86SNAYDDKSYENVEFIFRL67-LADDMPATP3DQEMYQPDPEMESR-TRYFSPRL480. thyellinaSNDVKDGGADGAH3DRGG-MFGPRL71VI FIFKL86-SSAYDDKSYENVEFIFRL61-LADDMPATP3DQEMYRDPEQIDSR-TKYFSPRL491. transeraNNNDKD-GAASGAH3DRG-LMFGPRL71VI FIFKL86-SSAYDDKSYENVEFIFRL61-LSDMPATPADQEMYRDPEQIDSR-TKYFSPRL411. transeraNNNDKD-GAASGAH3DRG-LMFGPRL67VI FIFKL100-SLSYDDKSFENVEFIFRL61-LSDMPATPADQEMYRDPEQIDSR-TKYFSPRL411. transeraNNNDKD-GAASGAH3DRG-LMFGPRL67VI FIFKL100-SLSYDDKSFENVEFIFRL61-LSDMPATPADQEMYRDPEQIDSR-TKYFSPRL411. transeraNNNDKD-GAASGAH3DRG-LMFGPRL67VI FIFKL100-SLSYDDKSFENVEFIFRL61-LSDMPATPADQEMYRDPEQIDSR-TKYFSPRL41	М.	vitrata	LDDSKD-EADRGA-SDRGT-LWFGPRL	92	VVFTPKL	86	SIGGVFQDKKYDNVEFIPRL	47	-IPDALPVTPSDDDVYSFKPDSGEVDRR-TSYFNPRL	60	KVSFSPRL	75
M. sextaSNDIKD-GEDGRAHSDRGA-IMFGRAFI79VIFTPEI86SIGDIYQE-KRYYENFEFTPRI55ISEDMPATPSDQEYPMYHPDPEQUDTR-TRYFSPRI40A. pernyiSNDIKD-EGDKGAHSDRGS-IMFGPRI75VIFTPKI100SIDDSTQEKRYFYENFEFTPRI75JLSDDMPATPADQE-MYQPDPEQEESR-TRYFSPRI43B. moriTDMO-ESDRGAHSERGA-IMFGPRI76IFTFKI86SVAKPQTHSLEFTPRI67JLSDMPATPADQE-MYQPDPEQESR-TRYFSPRI42C. anastomosisINNDNTMCDGADRGAHSDRGG-MFGPRI71VIFTPKI86SVAKPQTHOSLEFTPRI56LADDTPATPADQE-MYRQDPEQIDSR-SNYFSPRI430. dhyellinaSSNOVKDGADRGAHSDRGGUHFGPRI71VIFTPKI100-SLSYEDKMEDNVEFTPRI56LSDDMPATPADQE-MYRQDPEQIDSR-TKYFSPRI411. armigeraNNNDVKD-GAASGAHSDRGUHFGPRI71VIFTPKI100-SLSYEDKMEDNVEFTPRI51LSDDMPATPADQE-MYRQDPEQIDSR-TKYFSPRI511. asaultaNNNDVKD-GAASGAHSDRL-IMFGPRI67VIFTPKI100-SLSYDDKSFENVEFTPRI61LSDDMPATPADQE-MYRQDPEQIDSR-TKYFSPRI411. virescensNNNDVKD-GAASGAHSDRL-IMFGPRI67VIFTKI100-SLSYDDKSFENVEFTPRI61LSDDMPATPADQE-MYRQDPEQIDSR-TKYFSPRI512. siguaNNNDVKD-GAASGAHSDRL-IMFGPRI67VIFTKI100-SLSYDDKSFENVEFTRI61LSDDMPATPADQE-MYRQDPEQIDSR-TKYFSPRI513. siguaNNNDVKD-GAASGAHSDRL-IMFGPRI67VIFTKI100-SLAYDDKSFENVEFTRI61LSDDMPATPADQE-MYRQDPEQIDSR-TKYFSPRI514. sigua <th>s.</th> <th>c. ricini</th> <th>TNDVKD-EGDRGAHSDRGS-LWFGPRL</th> <th>79</th> <th>VIFTPKL</th> <th>100</th> <th>RASNAYQE-KRTYENVEFTPRL</th> <th>70</th> <th>-LTEDMPATPTDQEMFDQDPEQIDTR-TRYFSPRL</th> <th>42</th> <th>TMTFSPRL</th> <th>75</th>	s.	c. ricini	TNDVKD-EGDRGAHSDRGS-LWFGPRL	79	VIFTPKL	100	RASNAYQE-KRTYENVEFTPRL	70	-LTEDMPATPTDQEMFDQDPEQIDTR-TRYFSPRL	42	TMTFSPRL	75
A. pernyiSNDIKD-EGDKGAHSDRGS-IMFGPER75VIFTPK100SLDDSTQEKRVFYENFEFTPR75-LSDDMPATPKDQEMYPQDPQEQDTR-TRYFSPR16B. moriTDMGD-ESDRGAHSBRGA-IMFGPR76IFTFK86SVAKPQTHESLEFTPR50-LSEDMPATPADQEMYQDPDEEESSR-TRYFSPR42C. anastomosiTDMGD-ESDRGAHSBRGA-IMFGPR71VIFTK86SVAKPQTHSLEFTPR67-LSEDMPATPADQEYYRQDPQLDSR-SNYFSPR43A. ipsilonSNNDVKDGQADRGAHSDRGG-MFGPR71VIFTK100-SLSYEDKMEDNVEFTPR56LADDTPATPADQEYYRQDPQLDSR-TKYFSPR43A. ipsilonSNNDVKDGQADRGAHSDRGGUHFGPR71VIFTK100-SLSYEDKMEDNVEFTPR58LSDDMPATPADQEMYRQDPQLDSR-TKYFSPR43B. armigeraNNNDVKD-GAASGAHSDRG-IMFGPR67VIFTK100-SLSYEDKSFENVEFTR61LSDDMPATPADQEMYRQDPQLDSR-TKYFSPR41B. asmltaNNNDVKD-GAASGAHSDRJG-IMFGPR67VIFTK100-SLSYDDKSFENVEFTR61LSDDMPATPADQEMYRQDPQLDSR-TKYFSPR41F. virescensNNNDKKD-GAASGAHSDRJG-IMFGPR67VIFTK100-SLAYDDKSFENVEFTR61LSDDMPATPADQEMYRQDPQLDSR-TKYFSPR41S. ariguaNNNEIKDGGSDRGAHSDRG-IMFGPR67VIFTK100-SLAYDDKSFENVEFTR61LSDDMPATPADQEMYRQDPQLDSR-TKYFSPR41S. ariguaNNNEIKDGGSDRGAHSDRG-IMFGPR67VIFTK100-SLAYDDKSFENVEFTR61LADDMPATPADQELYRQDPQLDSR-TKYFSPR42S. ariguaNNNEIKDGGSDRGAHSDRG-IM	М.	sexta	SNDIKD-EGDRGAHSDRGA-LWFGPRL	79	VIFTPEL	86	SIGDIYQE-KRTYENVEFTPRL	55	-ISEDMPATPSDQEYPMYHPDPEQIDTR-TRYFSPRL	40	THFSPRL	75
B. mori    TUMACD-ESDRGAHSERGA-IMFGRPER     78     IIFTPK     86    SVAKPQTHESLEFIRR     50     LSEDMPATPADQEMYQPDPEMESR-TRYFSPR     42       B. mandarina    TUMACD-ESDRGAHSERGA-IMFGRPER     70     IIFTPK     86    SVAKPQTHDSLEFIRR     44     LSEDMPATPADQEMYQPDPEMESR-TRYFSPR     43       C. mastomosis     TUMDYTMCGGARGAHSDRGG-IMFGRPER     70     VIFTPK     80    SVAKPQTHDSLEFIRR     67     LADDMPATPADQEMYRQDPQEQDSR-SMYFSPR     43       A. ipsilon    SUNVKDGGARGAHSDRGG-IMFGRPER     71     VIFTPK     80     -SLSYEDKMEDNVEFTPR     61     LSDDMPATPADQEMYRQDPQLDSR-SMYFSPR     43       A. ipsilon    SUNVKDGGARGAHSDRGG/IMFGRF     71     VIFTPK     100     SLSYEDKMEDNVEFTPR     61     LSDDMPATPADQEMYRQDPQLDSR-TKYFSPR     63       A. armigera    NNDVKD-GAASGAHSDRJCH/MFGRF     67     VIFTPK     100     SLSYEDKSFENVEFTPR     61     LSDDMPATPADQEMYRQDPQLDSR-TKYFSPR     61       A. assulta    NNNDVKD-GAASGAHSDRJCH/MFGRF     67     VIFTPK     100     SLSYEDKSFENVEFTPR     61     LSDDMPATPADQEMYRQDPQLDSR-TKYFSPR     61     LSDDMPATPADQEMYRQDPQLDSR-TKYFSPR     61       G. sinsusta     -	A.	pernyi	SNDIKD-EGDKGAHSDRGS-LWFGPRL	75	VIFTPKL	100	SLDDSTQEKRVFYENFEFTPRL	75	-LSDDMPATPKDQEMYHQDPEQVDTR-TRYFSPRL	36	TITFSPRL	75
B. mandarina    TUMKD-ESDRGAHSERGA-IMFGPEN     78     IIFTPK     86    SVAKPQTHDSLEFIPR     44     -LSEDMPATPADQE-IYQPDPEVMESR-TRYFSPR     42       C. anastomosis     TNNDNTMCGGADRGAHSDRGG-IMFGPRI     75     VFTFK     86    SMAYDDKSYENVEFTPRI     67     IADDPATPADQE-IYQPDPEVMESR-TRYFSPRI     43       A. ipsilon    SSNDVKDGGADRGAHSDRGGUMFGPRI     71     VIFTKI     100     -SLSYEDKMFDNVEFTPRI     50     IADDTATPADQE-IYRQDPEQIDSR-TKYFSPRI     42       A. ipsilon    TONDVKDGQARVAHSDRGGUMFGPRI     67     VIFTKI     100     -SLSYEDKMFDNVEFTPRI     61     ISDDMPATPADQE-IYRQDPEQIDSR-TKYFSPRI     42       A. armigera    NNNDVKD-GAASGAHSDRLG-LWFGPRI     67     VIFTKI     100     -SLSYEDKSFENVEFTRI     61     LSDDMPATPADQE-IYRQDPEQIDSR-TKYFSPRI     41       A. assulta    NNNDVKD-GAASGAHSDRLG-LWFGPRI     67     VIFTKI     100     -SLAYDDKSFENVEFTRI     61     LSDDMPATPADQE-IYRQDPEQIDSR-TKYFSPRI     41       S. arigua    NNNDKKD-GAASGAHSDRLG-LWFGPRI     67     VIFTKI     100     -SLAYDDKSFENVEFTRI     61     LSDDMPATPADQE-IYRQDPEQIDSR-TKYFSPRI     42       S. arigua    NNNELKDGGSDRGAHSDRG-LWFGPRI     67 <td< td=""><td>в.</td><td>mori</td><td>TDMKD-ESDRGAHSERGA-LWFGPRL</td><td>78</td><td>IIFTPKL</td><td>86</td><td>SVAKPQTHESLEFIPRL</td><td>50</td><td>-LSEDMPATPADQEMYQPDPEEMESR-TRYFSPRL</td><td>45</td><td>TMSFSPRL</td><td>75</td></td<>	в.	mori	TDMKD-ESDRGAHSERGA-LWFGPRL	78	IIFTPKL	86	SVAKPQTHESLEFIPRL	50	-LSEDMPATPADQEMYQPDPEEMESR-TRYFSPRL	45	TMSFSPRL	75
C. anastomosis     TNNDNTMKDGADARDAHSDRGG-IMFGPRI     75     VVFTPKI     86     -SMAYDDKSYENVEFTPRI     67     IADDMPATPSDQEYYRQDPQQIDSR-SNYFSPRI     45       A. ipsilon    SSNDVKDGGADRGAHSDRGG-MMFGPRI     71     VIFTPKI     100     -SLSYEDKMEDNVEFTPRI     56     IADDTPATPADQEMYRDDPQQIDSR-TKYFSPRI     42       0. thyellina    TGNDVKDGQARVAHSDRGGUMFGPRI     67     VIFTPKI     100     -SLSYEDKMEDNVEFTPRI     51     ISDDMPATPADQEMYRDDPQQIDSR-TKYFSPRI     42       H. armigera    NNNDVKD-GAASGAHSDRIG-IMFGPRI     67     VIFTPKI     100     -SLSYDDKSFENVEFTPRI     61     ISDDMPATPADQEMYRQDPQUDSR-TKYFSPRI     41       H. asaulta    NNNDVKD-GAASGAHSDRIG-IMFGPRI     67     VIFTPKI     100     -SLSYDDKSFENVEFTPRI     61     ISDDMPATPADQEMYRQDPQUDSR-TKYFSPRI     41       H. virescens    NNNDVKD-GAASGAHSDRIG-IMFGPRI     67     VIFTPKI     100     -SLAYDDKSFENVEFTPRI     61     ISDDMPATPADQEMYRQDPQUDSR-TKYFSPRI     41       S. exigua    NNNDKDC-GAASGAHSDRIG-IMFGPRI     67     VIFTPKI     100     -SLAYDDKSFENVEFTPRI     61     ISDDMPATPADQELYRQDPQUDSR-TKYFSPRI     42       S. arigua     -NNNEIKDGGSDRGAHSDRG-IMFGPRI <td< td=""><td>в.</td><td>mandarina</td><td>TDMKD-ESDRGAHSERGA-LWFGPRL</td><td>78</td><td>IIFTPKL</td><td>86</td><td>SVAKPQTHDSLEFIPRL</td><td>44</td><td>-LSEDMPATPADQEIYQPDPEVMESR-TRYFSPRL</td><td>42</td><td>TMSFSPRL</td><td>75</td></td<>	в.	mandarina	TDMKD-ESDRGAHSERGA-LWFGPRL	78	IIFTPKL	86	SVAKPQTHDSLEFIPRL	44	-LSEDMPATPADQEIYQPDPEVMESR-TRYFSPRL	42	TMSFSPRL	75
A. ipsilon    SSNDVKDGQADRGAHSDRGG-MMFGPRI     71     VIFTPKI     100    SLSYEDKMFDNVEFTPRI     56     -LADDTPATPADQEMYRPDPQQLDSR-TKYFSPRI     12       0. thyellina    TGNDVKDGQQRVAHSDRGQQIMFGPRI     71     VIFTPKI     100     -SLSYEEKLYDNVEFTPRI     58     -LSDDMPATPADQEMYRPDPQQLDSR-TKYFSPRI     12       H. armigera    NNNDVKD-GAASGAHSDRLG-LMFGPRI     67     VIFTPKI     100     -SLSYDDKSFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPQLDSR-TKYFSPRI     41       H. asaulta    NNNDVKD-GAASGAHSDRLG-LMFGPRI     67     VIFTPKI     100     -SLSYDDKSFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPQLDSR-TKYFSPRI     41       H. asaulta    NNNDVKD-GAASGAHSDRLG-LMFGPRI     67     VIFTPKI     100     -SLAYDDKSFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPQLDSR-TKYFSPRI     41       S. arigua    NNNDKD-GAASGAHSDRLG-LMFGPRI     67     VIFTPKI     100     -SLAYDDKYFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPQLDSR-TKYFSPRI     42       S. arigua    NNNELKDGGSDRGAHSDRG-LMFGPRI     67     VIFTFKI     100     -SLAYDDKYFENVEFTPRI     61     -LSDDMPATPADQELYRPDPQDQISR-TKYFSPRI     42       S. littoralis    NNNELKDGGSDRGAHSDRAG-LMFGPRI	C.	anastomosis	TNNDNTMKDGGADRGAHSDRGG-LWFGPRL	75	VVFTPKL	86	SMAYDDKSYENVEFTPRL	67	-LADDMPATPSDQEYYRQDPEQIDSR-SNYFSPRL	45	TMTLTPRL	50
0. thyollina    TGNDVKDGQQDVAHSDRGQQIMFGPRI     71     VIFTPKI     100     -SLSTYEEKLYDNVEFTPRI     58     -LSDDMPATPPDQEYYRPDPQQLDSR-TKYFSPRI     42       R. axmigera    NNNDVKD-GAASGAHSDRLG-IMFGPRI     67     VIFTPKI     100     -SLSYDDKSFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPQQLDSR-TKYFSPRI     41       H. aca    NNNDVKD-GAASGAHSDRLG-IMFGPRI     67     VIFTFKI     100     -SLSYDDKSFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPQQLDSR-TKYFSPRI     41       H. aca    NNNDVKD-GAASGAHSDRLG-IMFGPRI     67     VIFTKI     100     -SLAYDDKSFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPQUDSR-TKYFSPRI     41       K. acsulta    NNNDKD-GAASGAHSDRLG-IMFGPRI     67     VIFTKI     100     -SLAYDDKSFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPQUDSR-TKYFSPRI     41       S. arigua    NNNEIKDGGSDRGAHSDRAG-IMFGPRI     67     VIFTKI     100     -SLAYDDKYFENVEFTRI     61     -LSDDMPATPADQELYRQDPQUDSR-TKYFSPRI     42       S. littoralis    NNNEIKDGGSDRGAHSDRAG-IMFGPRI     67     VIFTKI     100     -SLAYDDKYFENVEFTRI     61     -LSDDMPATPADQELYRQDPQLDSR-TKYFSPRI     42       A. S. oretacea    NNEIKDGGSDRGAHSDRAG-IMFGPRI	Α.	ipsilon	SSNDVKDGGADRGAHSDRGG-MWFGPRI	71	VIFTPKL	100	SLSYEDKMFDNVEFTPRL	56	-LADDTPATPADQEMYRPDPEQIDSR-TKYFSPRL	39	TMNFSPRL	88
H. armigera    NNNUVKD-GAASGAHSDRLG-LMFGPRI     67     VIFTPKI     100     -SLAYDDKSFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPEQLDSR-TKYFSPRI     41       H. sea    NNNUVKD-GAASGAHSDRLG-LMFGPRI     67     VIFTPKI     100     -SLSYDDKSFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPEQLDSR-TKYFSPRI     41       H. assulta    NNNUVKD-GAASGAHSDRLG-LMFGPRI     67     VIFTPKI     100     -SLAYDDKSFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPEQLDSR-TKYFSPRI     41       K. assulta    NNNUKD-GAASGAHSDRLG-LMFGPRI     67     VIFTPKI     100     -SLAYDDKSFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPEQLDSR-TKYFSPRI     41       S. arigua    NNNEIKDGSGSDRGAHSDRAG-LMFGPRI     67     VIFTPKI     100     -SLAYDDKVFENVEFTPRI     61     -LSDDMPATPADQEMYRQDPEQLDSR-TKYFSPRI     42       S. littoralis    NNNEIKDGSGSDRGAHSDRAG-LMFGPRI     67     VIFTPKI     100     -SLAYDDKVFENVEFTPRI     61     -LSDDMPATPADQELYRPDPDQLDSR-TKYFSPRI     42       A. S. cretacea    NNEIKDGGSDRGAHSDRAG-LMFGPRI     67     VIFTPKI     100     -SLAYDDKVFENVEFTPRI     61     -LADDMPATPADQELYRQDEQLISSR-TKYFSPRI     42       F. xylostella    NNEIKENGGSDRGAHSDRAG-LMFGP	0.	thyellina	TGNDVKDDGQDRVAHSDRGGQLWFGPRL	71	VIFTPKL	100	-SLSTYEEKLYDNVEFTPRL	58	-LSDDMPATPPDQEYYRPDPEQIDSR-TKYFSPRL	42	TMTFSPRL	75
H. zea    NNNDVKD-GAASGAHSDRLG-LMFGPER     67     VIFTPK     100    SLSYDDKSFENVEFTPR     61     -LSDDMPATPADQEMYRQDPQQLDSR-TKYFSPR     41       H. assulta    NNNDVKD-GAASGAHSDRLG-LMFGPRL     67     VIFTPK     100     -SLAYDDKSFENVEFTPR     61     -LSDDMPATPADQEMYRQDPQQLDSR-TKYFSPRL     41       H. assulta    NNNDKDG-GAASGAHSDRLG-LMFGPRL     67     VIFTPK     100     -SLAYDDKVFENVEFTPRL     61     -LSDDMPATPADQEMYRQDPQLDSR-TKYFSPRL     42       S. axigua    NNNEIKDGGSDRGAHSDRAG-LWFGPRL     67     VIFTPKL     100     -SLAYDDKVFENVEFTPRL     61     -LSDDMPATPADQELYRPDPDQLDSR-TKYFSPRL     42       S. littoralis    NNNEIKDGGSDRGAHSDRAG-LWFGPRL     67     VIFTPKL     100     -SLAYDDKVFENVEFTPRL     61     -LADDMPATPADQELYRPDPDQLDSR-TKYFSPRL     42       A. S. cretacea    ENFKEENFDRNIRSGRAN-VVFKPIL     67     VIFTPKL     100     -SLAYDDKVFENVEFTPRL     61     LADDMPATPADQELYRPDPDQLDSR-TKYFSPRL     42       F. xylostella    ENFKEENFDRNIRSGRAN-VVFKPIL     65     VIFTPKL     100     SMEDPYEEKRSYDVDFTPRL     32     RLKOSGLAPPLYRPDPQLIDSR-TKYFSPRL     41       Adoxophyees sp.    DLKE-EDGEREANSDRQG-LWFGPRL	Н.	armigera	NNNDVKD-GAASGAHSDRLG-LWFGPRL	67	VIFTPKL	100	SLAYDDKSFENVEFTPRL	61	-LSDDMPATPADQEMYRQDPEQIDSR-TKYFSPRL	39	TMNFSPRL	88
H. assulta    NNNDVKD-GAASGAHSDRLG-LMFGPRL     67     VIFTPKL     100    SLAYDDKSFENVEFTPRL     61     -LSDDMPATPADQEMYRQDPQQLDSR-TKYFSPRL     41       H. virescens    NNNDKKD-GAASGAHSDRLG-LMFGPRL     67     VIFTPKL     100     -SLAYDDKSFENVEFTPRL     61     -LADDMPATPADQEMYRQDPQQLDSR-TKYFSPRL     42       S. exigua    NNNEIKDGGSDRGAHSDRAG-LWFGPRL     67     VIFTPKL     100     -SLAYDDKVFENVEFTPRL     61     -LSDDMPATPADQELYRPDPQDIDSR-TKYFSPRL     42       S. littoralis    NNNEIKDGGSDRGAHSDRAG-LWFGPRL     67     VIFTPKL     100     -SLAYDDKVFENVEFTPRL     61     -LADDMPATPADQELYRPDPQDIDSR-TKYFSPRL     42       A. S. cretacea    ENFKEENFDRINIRSGRAN-VVFKPIL     65     VIFTPKL     100     -SLAYDDKVFENVEFTPRL     61     -LADDMPATPADQELYRPDPQDIDSR-TKYFSPRL     42       P. xylostella    ENFKEENFDRINIRSGRAN-VVFKPIL     65     VIFTPKL     100     -SLAYDDKVFENVEFTPRL     38     RLKDSGLAPPVRPQEPQDIDSR-TKYFSPRL     41       Adoxophyee sp.    ENFKEENFDRINIRSGRAN-VVFKPIL     61     VIFTPKL     100     -SLAYDDKVFENVEFTPRL     50     RLKDSGLAPPVPCPQENVEFTPRL     38     RLKDSGLAPPVPCPGRLAPGPCPL-SLYFFRL     41 </td <td>Н.</td> <td>zea</td> <td>NNNDVKD-GAASGAHSDRLG-LWFGPRL</td> <td>67</td> <td>VIFTPKL</td> <td>100</td> <td>SLSYDDKSFENVEFTPRL</td> <td>61</td> <td>-LSDDMPATPADQEMYRQDPEQIDSR-TKYFSPRL</td> <td>41</td> <td>TMNFSPRL</td> <td>88</td>	Н.	zea	NNNDVKD-GAASGAHSDRLG-LWFGPRL	67	VIFTPKL	100	SLSYDDKSFENVEFTPRL	61	-LSDDMPATPADQEMYRQDPEQIDSR-TKYFSPRL	41	TMNFSPRL	88
H. virescens    NNNDDKD-GAASGAHSDRLG-LMFGPL     67     VIFTPKL     100    SLAYDDKSFENVEFTPRL     61     -LADDMPATPADQEMYRQDPQQLDSRRTKYFSPRL     42       S. exigua    NNNEIKDGGSDRGAHSDRAG-LMFGPRL     67     VIFTPKL     100     -SLAYDDKVFENVEFTPRL     61     -LSDDMPATPADQELYRPDPDQLDSR-TKYFSPRL     42       S. littoralis    NNNEIKDGGSDRGAHSDRAG-LWFGPRL     67     VIFTPKL     100     -SLAYDDKVFENVEFTPRL     61     -LSDDMPATPADQELYRPDPDQLDSR-TKYFSPRL     42       A. S. cretacea    ENFKEENFDRNIRSGRAN-VVFKPIL     65     VIFTPKL     100    SLAYDDKVFENVEFTPRL     61     -LADDMPATPADQELYRPDPDQLDSR-TKYFSPRL     42       P. xylostella    ENFKEENFDRNIRSGRAN-VVFKPIL     65     VIFTPKL     100    SVDFTPRL     75     QLVDVPQ	Н.	assulta	NNNDVKD-GAASGAHSDRLG-LWFGPRL	67	VIFTPKL	100	SLAYDDKSFENVEFTPRL	61	-LSDDMPATPADQEMYRQDPEQIDSR-TKYFSPRL	41	TMNFSPRL	88
S. exigua    NNNEIKDGGSDRGAHSDRAG-IMFGPRI     67     VIFTPKI     100    SLAYDDKVFENVEFTPRI     61     -LSDDMPATPADQELYRPDPDQLDSR-TKYFSPRI     42       S. littoralis    NNNEIKDGGSDRGAHSDRAG-IMFGPRI     67     VIFTPKI     100    SLAYDDKVFENVEFTPRI     61     -LADDMPATPADQELYRPDPDQLDSR-TKYFSPRI     42       A. S. cretacea    ENFKEENFDRNIRSGRAN-VVFKPII     65     VIFTPKI     100    SVDFTPRI     75     QLVDDVPQRQQIEEDRLGSR-TRFFSPRI     36       P. xylostella    DDLKD-EDIQRDARDRAS-MWFGPRI     61     VIFTPKI     100     SMEDPYEEKRSYDVDFTPRI     38     RLKDSGLAPPDEYRTPELLDAR-AQYFSPRI     41       Adoxophyees sp.    NDLKE-DGEREANSDRQG-LWFGPRI     35     VIFTFKI     100    NADEDQQQSVDFTPRI     42    QEAVTSSDEQVYRQDMSPVDGR-LKYFSPRI     55	Н.	virescens	NNNDDKD-GAASGAHSDRLG-LWFGPRL	67	VIFTPKL	100	SLAYDDKSFENVEFTPRL	61	-LADDMPATPADQEMYRQDPEQIDSRRTKYFSPRL	41	TMNFSPRL	88
S. littoralis    NNNEIKDGGSDRGAHSDRAG-IMFGPRL     67     VIFTPKL     100    SLAYDDKVFENVEFTPRL     61     -LADDMPATPADQELYRPDPDQLDSR-TKYFSPRL     42       A. S. oretacea    ENFKEENFDRNIRSGRAN-VVFKPIL     65     VIFTPKL     100    SVDFTPRL     75     QLVDDVPQRQQLEEDRLGSR-TRFFSPRL     36       P. xylostella    DDLKD-EDIQRDARDRAS-MMFGPRL     61     VIFTPKL     100     SMEDPYEEKRSYDVDFTPRL     38     RLKDSGLAPPDEYRTPELLDAR-AQYFSPRL     41       Adoxophyees sp.    NDLKE-DGEREANSDRQG-IMFGPRL     35     VIFTPKL     100    NADEDQQQSVDFTPRL     42    QSEAVTSSDEQVYRQDMSPVDGR-LKYFSPRL     35	s.	exigua	NNNEIKDGGSDRGAHSDRAG-LWFGPRL	67	VIFTPKL	100	SLAYDDKVFENVEFTPRL	61	-LSDDMPATPADQELYRPDPDQIDSR-TKYFSPRL	42	TMNFSPRL	88
A. S. cretacea    ENFKEENFDRNIRSGRAN-VVFKPIL     65     VIFTPKL     100    SVDFTPRL     75     QLVDDVPQRQQLEEDRLGSR-TRFFSPRL     36       P. xylostella    DDLKD-EDIQRDARDRAS-MWFGPRL     61     VIFTPKL     100     SMEDPYEEKRSYDVDFTPRL     38     RLKDSGLAPPDEYRTPELLDAR-AQYFSPRL     41       Adoxophyes sp.    NDLKE-DGEREANSDRQG-LWFGPRL     35     VIFTPKL     100    NADEDQQQSVDFTPRL     42    QSEAVTSSDEQVYRQDMSPVDGR-LKYFSPRL     35	s.	littoralis	NNNEIKDGGSDRGAHSDRAG-LWFGPRL	67	VIFTPKL	100	SLAYDDKVFENVEFTPRL	61	-LADDMPATPADQELYRPDPDQIDSR-TKYFSPRL	42	TMNFSPRL	88
P. xylostella    DDLKD-EDIQRDARDRAS-MWFGPRL     61     VIFTPKL     100     SMEDPYEEKRSYDVDFTPRL     38     RLKDSGLAPPDEYRTPELLDAR-AQYFSPRL     41       Adoxophyes sp.    NDLKE-DGEREANSDRQG-LWFGPRL     35     VIFTPKL     100    NADEDQQQSVDFTPRL     42    QSEAVTSSDEQVYRQDMSPVDGR-LKYFSPRL     35	Α.	S. cretacea	ENFKEENFDRNIRSGRAN-VVFKPIL	65	VIFTPKL	100	SVDFTPRL	75	QLVDDVPQRQQIEEDRLGSR-TRFFSPRL	36	-TTMNFSPRL	88
Adoxophyes spNDLKE-DGEREANSDRQG-LWFGPRL 35 VIFTPKL 100NADEDQQQSVDFTPRL 42QSEAVTSSDEQVYRQDMSPVDGR-LKYFSPRL 35	P.	xylostella	DDLKD-EDIQRDARDRAS-MWFGPRL	61	VIFTPKL	100	SMEDPYEEKRSYDVDFTPRL	38	RLKDSGLAPPDEYRTPELLDAR-AQYFSPRL	41	GGSMTFSPRL	63
	Add	oxophyes sp.	NDLKE-DGEREANSDRQG-LWFGPRL	35	VIFTPKL	100	NADEDQQQSVDFTPRL	42	QSEAVTSSDEQVYRQDMSPVDGR-LKYFSPRL	35	TVKLTPRL	63

Figure 3.2 Schematic drawing of the DH-PBAN precursor polyprotein in *O. fuscidentalis*. (A) *DH-PBAN* cDNA encoding pre-prohormone consisting of 199 amino acids. It is presumed that the pre-prohormone undergoes post-translational processing via a series of enzymatic steps that cleave the GKR, KK, GRR, and 3 GR sequences, and further modification by amidation at the C-terminal amino acid of the intermediate peptide substrates to yield the signal sequence (SS) and peptide hormones DH,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -SGNP, and PBAN, similar to other Lepidopteran DH-PBAN precursor polyproteins. (B) Sequence alignment of DH, PBAN and three additional neuropeptides from 18 lepidopteran species, in addition to *O. fuscidentalis*. The percentages represent the amino acid similarities compared to *O. fuscidentalis*, which are calculated following ClustalW2 alignment. The GenBank accession numbers of these sequences are: AFX71575 (*Maruca vitrata*), AAP41132 (*Samia cynthia ricini*), AAO18192 (*Manduca*)

sexta), AAR17699 (Antheraea pernyi), AAB24327 (Bombyx mori), AAM88285 (Bombyx mandarina), ABR04093 (Clostera anastomosis), CAA08774 (Agrotis ipsilon), BAE94185 (Orygia thyellina), AAL05596 (Helicoverpa armigera), AAA20661 (Helicoverpa zea), AAC64293 (Helicoverpa assulta), AAO20095 (Heliothis virescens), AAT64424 (Spodoptera exigua), AAK84160 (Spodoptera littoralis), BAF64458 (Ascotis selenaria cretacea), AAX99220 (Plutella xylostella), and AAK72980 (Adoxophyes sp.).

Table 3.1 Homology of the deduced amino acid sequence of the OfDH-PBAN from the bamboo borer, *O. fuscidentalis* with diapause hormone and pheromone biosynthesis activating neuropeptide (DH-PBAN) of other lepidopteran species.

Species	Identity (%)	Similarity (%)	Accession Number
Maruca vitrata	73.7	88.4	M9P2L6
Antheraea pernyi	63.6	86.4	Q6SYA3
Helicoverpa armigera	63.0	83.5	Q95UR4
Spodoptera exigua	61.9	84.3	Q6RKA1
Bombyx mori	61.3	82.9	H9IWL9
Plutella xylostella	53.9	77.5 BRS	Q2M4G0

## 3.1.2 Phylogenetic analysis

A phylogenetic tree was constructed using the neighbour-joining method (Fig. 3.3). *Omphisa fuscidentalis* clusters together with *Helicoverpa assulta*, *H. armigera*, *Helicoverpa zea*, *Heliothis virescens*, *Spodoptera exigua*, *Spodoptera littoralis* (Noctuidae), *Antheraea pernyi*, *Samia cynthia ricini* (Saturniidae) and *M. vitrata* (Crambidae) with 97-100% bootstrap support, forming a monophyly of the superfamilies Noctuoidea, Bombycoidea and Pyraloidea. The families Noctuidae, Lymantriidae and Notodontidae form a monophyletic clade comprising the superfamily Noctuoidea. The sister group to the Noctuoidea is the Bombycoidea, to which the families Saturniidae, Sphingidae and Bombycidae belong. The Geometroidea (represented by *Ascotis selenaria cretacea*) and the Pyraloidea form a trichotomy with

the Noctuoidea plus Bombycoidea clade. The sister taxon to the above clade is the Yponomeutoidea plus Tortricoidea. On the whole, the DH-PBAN protein sequence similarity is correlated with the basic taxonomic relationships among the species and infers the feasibility and sensitivity of the DH-PBAN gene sequences as a phylogenetic marker in the class Insecta.



Figure 3.3 Phylogenetic tree inferred from the known lepidopteran DH-PBAN amino acid sequences by the neighbour joining method. The neighbour joining tree was constructed using MEGA5 software (www.megasoftware.net) based on Dayhoff matrix (PAM) with 1000 bootstrap replicates. The number above branches shows the percentage bootstrap support above 50%. The scale on the bottom indicates the number of substitutions per amino acid site. The corresponding taxonomic families and superfamilies of the taxa and clades are shown on the right.

#### 3.2 OfDH-PBAN mRNA expression

#### 3.2.1 Tissue distribution and developmental expression of OfDH-PBAN

The expression of the *OfDH-PBAN* mRNA was examined by Quantitative realtime PCR. Total RNA was isolated from SG, other neural tissues (e.g., brain, thoracic ganglia and abdominal ganglia) and non-neural tissues (e.g., fat body, integument and Malpighian tubule). The results showed that *OfDH-PBAN* mRNA was expressed in the SG, detectable at much lower levels in other neural tissues, and not detected in the nonneural tissues (Fig. 3.4). Furthermore, the expression of the *OfDH-PBAN* transcript was detected during larval and pupal development. The expression level of *OfDH-PBAN* mRNA was consistently high during the 5<sup>th</sup> instar of larval development, then moderately high from October to December (larval stage) and significantly increased in January (larval stage), reaching the maximum level in March (larval stage). After pupation, the expression sharply decreased to a low level (Fig. 3.5).



Figure 3.4 Tissue expression of *OfDH-PBAN* mRNA from young 5<sup>th</sup> instar larvae of *O. fuscidentalis*. Total RNA (1.0  $\mu$ g) was isolated from various tissues and mRNA expression was determined by Q-RT-PCR. Suboesophageal ganglion (SG), brain (Br), thoracic ganglia (TG), abdominal ganglia (AG), Malpighian tubules (Mt), fat body (Ft) and integument (Inm). The data represent mean values of three independent samples, normalized relative to ribosomal protein RpL3 transcript levels. The integument was taken as the calibrator sample.



Figure 3.5 Developmental changes in the expression levels of *OfDH-PBAN* mRNA in the subesophageal ganglion of *O. fuscidentalis* during diapause (October to March) and post-diapause (pupation) as measured by Q-RT-PCR. The results are expressed as the relative expression after normalization against endogenous ribosomal protein mRNA *OfRpL3*. Expression is relative to the gene expression in diapausing larvae collected from October (assigned a value of 1). Each value is the mean  $\pm$  SEM of three independent experiments. Means with different letters are significantly difference (ANOVA, n = 3, P < 0.05). The dotted line represents the hemolymph ecdysteroid titer (Singtripop *et al.*, 1999).

## 3.2.2 Effect of photoperiod on OfDH-PBAN mRNA expression

Because there was an evidence that photoperiod induced pupation in diapausing *O. fuscidentalis* larvae, the effect of photoperiod on the expression of *OfDH-PBAN* mRNA by quantitative real-time PCR was also examined in the SG of larvae reared at 25 °C combined with various photoperiods (LD 0:24 h, LD 2:22 h, LD 14:10 h and LD 18:6 h). Results showed that the photoperiod had an effect on the expression level of *OfDH-PBAN* mRNA. Under the photocycles of LD 2:22 h, LD 14:10 h and LD 18:6 h during larval diapause the expression level *OfDH-PBAN* mRNA was low during the first twenty days of the larval stage. At day-25, the expression was significantly higher, and it dropped abruptly to a minimum in the pupal stage (Fig. 3.6).



Figure 3.6 Effect of diapause-inducing photoperiod on the expression of *OfDH-PBAN* gene in the suboesophageal ganglion of diapausing larvae of *O. fuscidentalis*. Larvae were reared at 25 °C under various photoperiods (LD 0:24 h, LD 2:22 h, LD 14:10 h, and LD 18:6 h). The *OfDH-PBAN* mRNA levels were determined from total RNA at various during diapause (D5-D25, day after feeding) and post-diapause (pupation) under each photocycle. Total RNA was isolated from the suboesophageal ganglion of staged *O. fuscidentalis* larvae, and mRNA levels were analyzed using Q-RT-PCR. The results are expressed as the relative expression after normalization against endogenous *OfRpL3*.The different controls that were collected at the same time points as treated samples were used as the calibrator sample. The data represent mean values of three independent experiments. See also the legend to Fig. 3.5.

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### 3.3 Molecular characterization of OfMet, OfE75C and OfHR3 cDNA

#### 3.3.1 Cloning of OfMet cDNA

The partial sequence of *OfMet* contains 748 nucleotides, encoding a protein of 248 amino acids. By homology search (Table 3.2), the deduced protein sequence of OfMet revealed the highest homology with *B. mori* (63%), followed by *D. plexippus* (62%), *O. brumata* (38%) and *H. armigera* (36%). We have confirmed the molecular structure similarity of known Met genes among species in the order Lepidoptera. The OfMet amino acid sequences were aligned with those of two other insect species, the protein sequence of OfMet is most homologous to Met from *B. mori* of the family Bombycidae and least similar to that of *O. brumata* in the Geometridae (Fig. 3.7).

Table 3.2 Homology of the deduced amino acid sequences of the OfMet from the bamboo borer, *O. fuscidentalis* with Methoprene tolerant (Met) of other lepidopteran species.

Bombyx mori	63.4	D4Q9H9
Dangus playinnus	MATTI	
Danaus piexippus	62.4	G6CT04
Operophtera brumata	37.7 FRS	A0A0L7LC43
Helicoverpa armigera	35.7	A0A023PPA4

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OfMet	$\tt DKTGALRLTAHYLRAHQYVFGDSIGQGNPQFSAMSARALLSLLKGFLLTTTYRGLIVVVS$	60
BmMet	DKTGVLRLTAHYLRAHQYVFCNKMVHTNPDFNPEFTDAVLKLFNGFLITTTYRGIIVVVS	60
ObMet	DKTSVLRLAANKLRNE-HVFGNTIKCSHIETWSPAFLKFFDLIGGIMFTVTCRGRICIIS	59
	******:*. ** . :** :.: . : .:.*: *::*.* ** * ::*	
OfMet	QNVQQYLGYTELDLLGQNVFNIIHEDDRQLMRDQLMPKKNMLGPNGELLVPEEPEGNRMV	120
BmMet	KNVHQYLGFPELDLLGQNLVNLTHPRDRQMLLEKLKPRSQVLGPNGELLIPNEPDGVYKV	120
ObMet	PNIQEKLGYCYVDLLGLDLYNYVHPDDKEILHQHIYPHELQTGSDSRL	107
	*::: **: :**** :: * * *:::: ::: *:. * :*	
OfMet	AQILAGEKRRFIIRFKKFCQRSEPCQYVTCHVEGTLRKSDRACRGYNRCCQMVRRARARG	180
BmMet	VEGLRREKRSFTIRLKKQGPRSEPAQYVMCHIEGSFRKADGANHTLSRCCQVVRRSRTRG	180
ObMet	YEQHHNFNIRIKRAGARSDPVRYERCRIDGMLRKSDKAIANAVQDERVIRRQRVRQ	163
	::: * **:*: **:* :* *:::* :**:* * : ::::** *.*	
	ab	
OfMet	D-NPCSSGNDIVFVGVVRVATETFITESNMESYRMEYRTRHSIDGQIIQSEQRISLVTGY	239
BmMet	E-APECSGNDIVFIGVVRPSVETFHSESRMESFCMEYRTRHSVDGQIVQCEQRISLVTGY	239
ObMet	NRTFSSSGNDFVFIGMIHVLSSNLPPRILPPTAYSEYWTRHMIDGRIVQCDQSISLAVGY	223
	: .***:*:::: . : ** *** :**:*:* .:* *****	
	A La Commence	
OfMet	MTHERARLV 248	
BmMet	MTHEVKGVN 248	
ObMet	MTDEVTGTS 232	
	**.*	

Figure 3.7 Clustal W2 alignment of deduced amino acid sequence of OfMet with sequences from two other insect species (BmMet, *B. mori* and ObMet, *O. brumata*). Number on the right side of the alignment indicates the position of the residues in the sequence of each protein. Conserved positions (identical amino acids) are asterisked.

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#### 3.3.2 Cloning of OfE75C cDNA

The partial sequence of *OfE75C* contains 449 nucleotides, encoding a protein of 149 amino acids. By homology search (Table 3.3), the deduced protein sequence of OfE75C revealed the highest homology with *B. mori* and *M. sexta* (96%), followed by *A. aegypti* (78%). We have confirmed the molecular structure similarity of known E75C genes among species in the order Lepidoptera. The OfE75C amino acid sequences were aligned with those of two other insect species, the protein sequence of OfE75C is most homologous to E75C from *B. mori* and *M. sexta* of the family Bombycidae and Sphingidae, respectively (Fig. 3.8).

Table 3.3 Homology of the deduced amino acid sequence of the OfE75C from the bamboo borer, *O. fuscidentalis* with E75C of other lepidopteran species.

-	Species	Identity (%)	Accession Number	
-	Bombyx mori	96.1	Q8WSA1	
	Manduca sexta	96.1	Q5YB71	
_	Aedes aegypti	77.5	Q0JRL0	
	17	× 1134	A	
OfE75C	SVIQCMRPPPPPP	PRLLKPSSFEEPSSSIPDLE	FDGTTVLCRVCGDKASGFHYGVH 57	
MsE75C	SVIQCMRPPPPPPP	PAPRLHKPPSFEEPSSSIPDLE	FDGTTVLCRVCGDKASGFHYGVH 60	
BmE75C	SVIQCMRPPPPPPP	PPRLLKPPSFEEPSSSIPDLE	FDGTTVLCRVCGDKASGFHYGVH 60	
	* * * * * * * * * * * * * *	*** ** **********	****	
	S. S. S.			
OfE75C	SCEGCKGFFRRSIQQ	XIQYRPCTKNQQCSILRINRNRO	CQYCRLKKCIAVGMSRDAVRFGR 11	7
MsE75C	SCEGCKGFFRRSIQQ	XIQYRPCTKNQQCSILRINRNRO	CQYCRLKKCIAVGMSRDAVRFGR 120	0
BmE75C	SCEGCKGFFRRSIQQ	XIQYRPCTKNQQCSILRINRNR(	CQYCRLKKCIAVGMSRDAVRFGR 120	0
	************	**************************************	eserved	
OfE75C	VPKREKARILAAMQQS	STSRAHEQAATAELDD 149	9	
MsE75C	VPKREKARILAAMQQS	STSRAHEQAAAAELD- 151	L	
BmE75C	VPKREKARILAAMQQS	SSSSRAHEQAAAAELD- 153	1	
	**********	*:****		

Figure 3.8 Clustal W2 alignment of deduced amino acid sequence of OfE75C with sequences from two other insect species (MsE75C, *M. sexta* and BmE75C, *B. mori*). Number on the right side of the alignment indicates the position of the residues in the sequence of each protein. Conserved positions (identical amino acids) are asterisked.

#### 3.3.3 Cloning of OfHR3 cDNA

The partial sequence of *OfHR3* contains 984 nucleotides, encoding a protein of 327 amino acids. By homology search (Table 3.4), the deduced protein sequence of OfHR3 revealed the highest homology with *P. xuthus* (89%), followed by *H. armigera* (83%), *B. mori* (81%), *P. interpunctella* (73%) and *C. fumiferana* (68%). We have confirmed the molecular structure similarity of known HR3 genes among species in the order Lepidoptera. The OfHR3 amino acid sequences were aligned with those of three other insect species, the protein sequence of OfHR3 is most homologous to HR3 from *P. xuthus*, *H. armigera* and *B. mori* of the family Papilionidae, Noctuidae and Bombycidae, respectively (Fig. 3.9).

Table 3.4 Homology of the deduced amino acid sequence of the OfHR3 from the bamboo borer, *O. fuscidentalis* with HR3 of other lepidopteran species.

Species	Identity (%)	Accession Number
Papilio xuthus	88.9	I4DLS9
Helicoverpa armigera	82.8	Q9BMC6
Bombyx mori	81.0	Q9U5G6
Plodia interpunctella	72.6	Q6PWP7
Choristoneura fumiferana	68.0	Q27547

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OfHR3	TVVNYQCPRNKACVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRF	60
PxHR3	TVVNYQCPRNKACVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRF	60
HaHR3	TVVNYQCPRNKACVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRF	60
BmHR3	TVVNYQCPRNKACVVDRVNRNRCQYCRLQKCLKLGMSRDAVKFGRMSKKQREKVEDEVRY	60
	***************************************	
OfHR3	${\tt HRAQMRAQTDAAPDSVYDAQQQTPSSSDQFHGHYNGYPAYGSPLSSYGYNNAGPALPSNM}$	120
PxHR3	${\tt HRAQMRAQTDAAPDSVYDAQQQTPSSSDQFHGHYNGYPGYSSPLSSYGYSGAGPALTSNM}$	120
HaHR3	${\tt HRAQMRAQTDTAPDSVYDAQQQTPSSSDQFHGHYNGYPGYGSPLSSYGYNNAGPALQSNM}$	120
BmHR3	${\tt HKAQMRVQADAAPDSVYDAQQQTPSSSDQFHGHYNSYPGYGSPLSSYGYNNAGPALPSNM}$	120
	* * * * * * * * * * * * * * * * * * * *	
OfHR3	SGMQAAPPQQQSYDVSADYVDST-TYEPKQ-TGFLDTDFIG 159	
PxHR3	N-IPPQQQQPQPYDVSADYVDSTTTYEPKQTGGFLDPDFIG 160	
HaHR3	GGIQPQAPQQQPYDVSADYVDSTTAYEPKQTEGFLDPDFIS 161	
BmHR3	SGMQPQPPAQPPYEVSGDYVDSTTTYEPKQ-TGFLDADFIS 160	
	· · · · · · · · · · · · · · · · · · ·	

Figure 3.9 Clustal W2 alignment of deduced amino acid sequence of OfHR3 with sequences from three other insect species (PxHR3; *P. xuthus*, HaHR3; *H. armigera* and BmHR3; *B. mori*). Number on the right side of the alignment indicates the position of the residues in the sequence of each protein. Conserved positions (identical amino acids) are asterisked.



# 3.4 *In vivo* effects of JHA on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in both SG and PG

We induced pupation by treating diapausing larvae with JHA and examined the effect of JHA on OfMet, OfDH-PBAN, OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, Of E75C and Of HR3 mRNA expression in both SG and PG (Fig. 3.10). After the application of 0.5 µg JHA, OfMet mRNA levels in the SG were low on day 0, while levels of OfMet mRNA in the PG were low until day 1. Thereafter, it then increased and remained until reaching a maximum level on day 8, decreased on day 10 and abruptly dropped to a minimum level until G3 stage (Fig. 3.10A). In addition, OfDH-PBAN mRNA levels in the SG were low from days 0 to 4, and then gradually increased until reaching a maximum level on day 12, while OfDH-PBAN mRNA levels in the PG were low from days 0 to 8, and then increased dramatically until reaching a peak on day 12. Expression of both SG and PG then abruptly dropped to a low level at G0-G3 stage (Fig. 3.10B). Interestingly, OfMet and OfDH-PBAN in the PG was expressed much later than OfMet and OfDH-PBAN in the SG. In the SG, the expression level of OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C and OfHR3 mRNA was expressed at a very low level between day 0 to G3 stage (Fig. 3.10C-I). By contrast, OfEcR-A and OfEcR-B1 mRNA levels in PG were low from days 0 to 12, and then increased dramatically to a high level at G0-G3 stage. The expression level of OfEcR-A mRNA reached a maximum level at G1 and G2 stage, but the OfEcR-B1 mRNA level peaked at G1 stage (Fig. 3.10C and D). In addition, the expression level of OfBr-C, OfE75A, OfE75B, OfE75C and OfHR3 mRNA in the PG was low between day 0 and G0 stage, then rapidly increased to the maximal value at the G1 stage and decreased at G2 and G3 stage (Fig. 3.10E-I). rights reserved

These results indicated that JHA had an effect on expression of *OfMet* and *OfDH-PBAN* mRNA in SG and PG. Interestingly, *OfMet* and *OfDH-PBAN* in the SG was expressed much faster than *OfMet* and *OfDH-PBAN* in the PG. Moreover, JHA affected on expression of *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA in PG during pupal stage.



Figure 3.10 Induction of JHA on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in SG and PG of diapausing larvae. Larvae were applied by 0.5 µg JHA, and *OfMet* (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and *OfHR3* (I) mRNA levels were determined from the total of pool RNA after treatment; D0-D12, 0-

12 days after treatment, G0-G3, stages in pupal development. The larvae became motionless before the formation of a brown cuticle (G0). The body colour then changes from creamy to light yellow (G1). The next day, the dorsal epidermis turns light brown (G2). Approximately 1 day later, the entire body becomes brown (G3) (Singtripop *et al.*, 2000). For the controls, diapausing larvae were applied by acetone. Total RNA was isolated from SG or PG of staged *O. fuscidentalis* larvae (as above) and mRNA levels were analyzed using the quantitative real-time polymerase chain reaction. The results are expressed as the relative expression after normalization against the expression of endogenous *OfRpL3*. Values are relative to the level of expression in the different controls collected at the same time points as the treated samples. Each value is the mean  $\pm$  SEM of three independent experiments. Means with different letters are significantly different (ANOVA, n = 3, P < 0.01).

# 3.5 In vivo effects of 20E on OfMet, OfDH-PBAN, OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C and OfHR3 mRNA expression in both SG and PG

We also examined the effects of 20E on the expression of *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA in both SG and PG (Fig. 3.11). In larvae injected with 0.5 µg 20E, the expression level of *OfMet* and *OfDH-PBAN* mRNA in both SG and PG was low from day 0 to G3 stage and was not significant when compared with control (Fig. 3.11A and B). Moreover, *OfEcR-A* and *OfEcR-B1* mRNA levels in the SG were low from days 0 to 10, and then increased gradually until reaching a maximum level at G3 stage, while *OfEcR-A* and *OfEcR-B1* mRNA levels in the PG were low on days 0 to 10, then increased to a maximum level in the G1 stage and decreased again between G2 and G3 stage (Fig. 3.11C and D). Furthermore, the expression level of *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA was low between days 0 and G0 stage, then increased dramatically and peaked at G1 stage, followed by a decline at G2 and G3 stage (Fig. 3.11E-I).

These results indicated that 20E had no effect on expression of *OfMet* and *OfDH-PBAN* mRNA in both SG and PG but had effect on expression of *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA in both SG and PG during pupal stage.



Figure 3.11 Induction of 20E on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in SG and PG of diapausing larvae. Larvae were injected with 0.5 µg of 20E, and *OfMet* (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and *OfHR3* (I) mRNA levels were determined from the total of pool RNA after treatment;

D0-D10, 0-10 days after treatment, G0-G3, stages in pupal development. The larvae became motionless before the formation of a brown cuticle (G0). The body colour then changes from creamy to light yellow (G1). The next day, the dorsal epidermis turns light brown (G2). Approximately 1 day later, the entire body becomes brown (G3) (Singtripop *et al.*, 2000). For the controls, diapausing larvae were injected by distilled water. Total RNA was isolated from SG or PG of staged *O. fuscidentalis* larvae (as above) and mRNA levels were analyzed using the quantitative real-time polymerase chain reaction. The results are expressed as the relative expression after normalization against the expression of endogenous *OfRpL3*. Values are relative to the level of expression in the different controls collected at the same time points as the treated samples. Each value is the mean  $\pm$  SEM of three independent experiments. See also the legend to Fig. 3.10.

# 3.6 *In vitro* effects of JHA and 20E on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in the SG

To understand how hormonal mechanism on termination of larval diapause is regulated by JHA, the SG was cultured in Grace's medium containing both JHA at 0.1  $\mu$ g/50  $\mu$ l and 20E at 0.1  $\mu$ g/50  $\mu$ l for different continuous time exposures. The induction patterns of *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression was examined under these experimental conditions.

We remarked that in the presence of JHA alone at 0.1  $\mu$ g/50  $\mu$ l, the *OfMet* mRNA levels were low at 0 h, and then rapidly increased to the maximal value at 0.5 h, followed by a decline from 1 to 24 h (Fig. 3.12A), while the expression level of *OfDH-PBAN* was low at 0 h, reached a maximum at 1 h and then decreased at 4-24 h (Fig. 3.12B). By contrast, JHA did not significantly up-regulate *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression (Fig. 3.12C-I). In the presence of 20E alone, *OfMet* and *OfDH-PBAN* mRNA levels were constitutively expressed at a low level over time (Fig. 3.12A and B) whereas the level of *OfEcR-A* and *OfEcR-B1* mRNA was low at 0 h, then dramatically increased at 0.5 h, reached the highest level at 1 h, followed by a large decrease at 4 h and remained steady at a low level until 24 h (Fig. 3.12C and D). The relative expression of *OfBr-C*, *OfE75A* and

OfHR3 was low between 0 and 0.5 h, then increased dramatically to the maximal value at 4 h and decreased at 8-24 h (Fig. 3.12E, F and I), while the OfE75B and OfE75C mRNA levels were low at 0-1 h, rapidly increased to a maximum level at 4 h, followed by a decline at 8 h and remained at a low level until 24 hour after 20E application (Fig. 3.12G and H). Moreover, we performed a time course experiment to determine how the presence of the two hormones together affected gene expression as a function of time. In the presence with 0.1 µg/50 µl of JHA and 20E together, OfMet and OfDH-PBAN transcripts showed temporal patterns similar to those obtained in response to JHA alone (Fig. 3.12A and B). The OfEcR-A and OfEcR-B1 mRNA levels were low at 0 and 1 h, and then increased until reaching a maximum level at 1 h, followed by a decline from 4 to 24 h (Fig. 3.12C and D). In addition, the expression level of OfBr-C, OfE75A and Of E75C mRNA was low between 0 and 0.5 h, while Of E75B and Of HR3 mRNA levels were low from 0 to 1 h. Thereafter, expression then increased until reaching a maximum level at 4 hour and declined at 8-24 h after application of the two hormones (Fig. 3.12E-I). These results indicated that JHA alone had effect on expression of OfMet and OfDH-PBAN mRNA (Fig. 3.12A-B), while 20E alone had effect on expression ecdysone receptor genes and ecdysone inducible genes in SG (Fig. 3.12C-I).

We next examined the dose-dependence on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression by adding various concentrations of JHA and 20E (0.005-1  $\mu$ g/50  $\mu$ l) to the medium. The total RNA was collected and isolated from the SG. On the basis of results of the time course experiment, the SG was incubated with various concentrations of JHA for 0.5 h (Fig. 3.13). The results showed that *OfMet* and *OfDH-PBAN* mRNA levels were low at 0.005  $\mu$ g/50  $\mu$ l JHA, then moderately high from 0.01-0.05  $\mu$ g/50  $\mu$ l JHA and reached a maximum at 0.1-1  $\mu$ g/50  $\mu$ l JHA (Fig. 3.13A and B), while *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA levels were constitutively expressed at a low level over dose of JHA (Fig 3.13C-I). By contrast, the SG was incubated with different concentrations of 20E for 4 h (based on the results of the time course experiment). 20E did not significantly up-regulate *OfMet* and *OfDH-PBAN* mRNA expression (Fig. 3.13A and B) whereas *OfEcR-A*, *OfEcR-B1*, *OfE75A*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA levels were low at 0.005  $\mu$ g/50  $\mu$ l 20E and gradually increased until reaching a maximum level at 0.1-1  $\mu$ g/50  $\mu$ l 20E (Fig. 3.13C-I).



Figure 3.12 Induction of JHA and 20E on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in the SG of diapausing larvae *in vitro*. Time course analysis of *OfMet* (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and

*OfHR3* (I) mRNA after treatment with 0.1 µg/50 µl of JHA alone, 0.1 µg/50 µl of 20E alone or 0.1 µg/50 µl of JHA and 20E together determined at 0-24 h. Total RNA was isolated and mRNA expression levels were determined using the quantitative real-time polymerase chain reaction. The results are expressed as the relative expression after normalization against endogenous *OfRpL3*. Expression is relative to the gene expression in the different controls (SG incubated in medium without hormonal additions were collected at the same time points as treated samples). Each value is the mean  $\pm$  SEM of three independent experiments. Means with different letters are significantly different (ANOVA, n = 3, P < 0.01).



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Figure 3.13 Concentration responses of *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA to JHA and 20E (from 0.005 to 1  $\mu$ g/50  $\mu$ l) in the SG of diapausing larvae *in vitro*. Dose response of *OfMet* (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and *OfHR3* (I) at 0.5 h after treatment with different concentrations of JHA and at 4 h after treatment with different concentrations of 20E. Total RNA was isolated and

mRNA expression levels were determined using the quantitative real-time polymerase chain reaction. The data represent mean values of three independent samples, normalized relative to *OfRpL3* transcript levels. 0.005  $\mu$ g/50  $\mu$ l of JHA alone or 0.005  $\mu$ g/50  $\mu$ l of 20E alone was taken as the calibrator sample.

# 3.7 *In vitro* effects of JHA and 20E on *OfDH-PBAN*, *OfMet*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in the PG

We also examined the effect of JHA and 20E on the change of OfMet, OfDH-PBAN, OfEcR-A, OfEcR-B1, OfBr-C, OfE75A, OfE75B, OfE75C and OfHR3 mRNA expression in PG, the PG was incubated in Grace's medium containing both 0.1 µg/50  $\mu$ l of JHA and 0.1  $\mu$ g/50  $\mu$ l of 20E as a function of time. In the presence of JHA alone, the results showed that the relative expression of OfMet mRNA was low at 0 h, rapidly increased at 0.5 h, peaked at 1 h and then decreased at 4-24 h, while OfDH-PBAN was low between 0 and 1 h, then increased suddenly to a maximum level at 4 h, followed by a decrease at 8 h and remained at low levels from 12-24 h (Fig 3.14A and B). Moreover, OfEcR-A and OfEcR-B1 mRNA levels were low from 0-4 h, and then increased dramatically to the maximal value at 8 h, followed by a decline at 12-24 h (Fig. 3.14C and D). In addition, the expression level of OfBr-C, OfE75A, OfE75B, OfE75C and OfHR3 mRNA was low from 0 to 8 h, and then increased rapidly to a maximum level at 12 h. Expression of OfBr-C, OfE75A, OfE75B, OfE75C and OfHR3 mRNA then declined and remained steady at a low level until 24 h after JHA addition (Fig. 3.14E-I). In the presence of 20E alone, OfMet and OfDH-PBAN mRNA levels remained at a low level at 0-24 h and were not significant when compared with control (Fig. 3.14A and B). By contrast, the relative expression of OfEcR-A and OfEcR-B1 mRNA was low at 0 h, then rapidly increased to a maximum level at 0.5 h and decreased at 1 h. Expression then declined abruptly to low levels until 24 h (Fig. 3.14C and D). The relative level of OfBr-C, OfE75A and OfE75B mRNA expression was low at 0 h, and then increased dramatically until reaching a maximum level at 1 h. Thereafter, it decreased at 4 h and then remained at low levels from 8-24 h (Fig. 3.14E, F and G). Of E75C and Of HR3 mRNA levels gradually increased and peaked at 4 h, followed by a decline at 8-24 h after the application of 20E (Fig. 3.14H and I). In combination of both JHA at 0.1  $\mu$ g/50 µl and 20E at 0.1 µg/50 µl for the time course experiment, OfMet and OfDH-PBAN

mRNA expression showed a similar pattern on those obtained in response to JHA alone (Fig 3.14A and B), while the relative level of *OfEcR-A* and *OfEcR-B1* mRNA was low at 0 h, then increased dramatically until reaching a maximum level at 0.5 h and gradually decreased to a low level (Fig. 3.14C and D). The expression level of *OfBr-C*, *OfE75A*, *OfE75B* and *OfE75C* mRNA increased gradually and peaked at 1 h whereas *OfHR3* mRNA levels increased gradually until reaching a maximum level at 8 h. Thereafter, expression of these five genes then gradually decreased to a low level after application of the two hormones (Fig. 3.14E-I).

These results indicated that JHA alone showed effect on expression of *OfMet* and *OfDH-PBAN* mRNA (Fig. 3.14A-B) whereas JHA and 20E had effect on expression of ecdysone receptor genes and ecdysone inducible genes (Fig. 3.14C-I). In addition, the increase in *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in PG cultured with both JHA and 20E was higher than PG incubated in medium contained either JHA or 20E. This observation suggested that the presence of both hormones induced an increase in expression level of these genes in PG (Fig. 3.14C-I).

We next examined the dose-dependence on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression by adding various concentrations of JHA and 20E (0.005-1  $\mu$ g/50  $\mu$ l) to the medium. The total RNA was collected and isolated from the PG. Based on the previous results of the time course experiment, the PG was incubated with various concentrations of JHA for 4 h (*OfMet* and *OfDH-PBAN*), for 8 h (*OfEcR-A* and *OfEcR-B1*) or for 12 h (*OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3*) (Fig. 3.15). The results showed that *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA levels were low at 0.005  $\mu$ g/50  $\mu$ l JHA, then gradually increased to a high level at 0.1-1  $\mu$ g/50  $\mu$ l JHA (Fig. 3.15A-I). Moreover, the PG was incubated with different concentrations of 20E for 1 h (based on the results of the time course experiment). 20E did not significantly up-regulate *OfMet* and *OfDH-PBAN* mRNA expression (Fig. 3.15A and B), while *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA levels were low at 0.005  $\mu$ g/50  $\mu$ l 20E, then gradually increased at 0.01 and 0.05  $\mu$ g/50  $\mu$ l 20E and peaked at 0.1-1  $\mu$ g/50  $\mu$ l 20E (Fig. 3.15C-I).



Figure 3.14 Induction of JHA and 20E on *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA expression in the PG of diapausing larvae *in vitro*. Time course analysis of *OfMet* (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and

*OfHR3* (I) mRNA after treatment with 0.1  $\mu$ g/50  $\mu$ l of JHA alone, 0.1  $\mu$ g/50  $\mu$ l of 20E alone or 0.1  $\mu$ g/50  $\mu$ l of JHA and 20E together determined at 0-24 h. Total RNA was isolated and mRNA expression levels were determined using the quantitative real-time polymerase chain reaction. The results are expressed as the relative expression after normalization against endogenous *OfRpL3*. Expression is relative to the gene expression in the different controls (PG incubated in medium without hormonal additions were collected at the same time points as treated samples). Each value is the mean  $\pm$  SEM of three independent experiments. See also the legend to Fig. 3.12.



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Figure 3.15 Concentration responses of *OfMet*, *OfDH-PBAN*, *OfEcR-A*, *OfEcR-B1*, *OfBr-C*, *OfE75A*, *OfE75B*, *OfE75C* and *OfHR3* mRNA to JHA and 20E (from 0.005 to 1  $\mu$ g/50  $\mu$ l) in the PG of diapausing larvae *in vitro*. The PG was incubated with different concentrations of JHA for 4 h to measure mRNA levels of *OfMet* (A) and *OfDH-PBAN* (B), for 8 h to measure *OfEcR-A* (C) and *OfEcR-B1* (D) or for 12 h to measure *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and *OfHR3* (I). Effect of 20E doses on

*OfMet* (A), *OfDH-PBAN* (B), *OfEcR-A* (C), *OfEcR-B1* (D), *OfBr-C* (E), *OfE75A* (F), *OfE75B* (G), *OfE75C* (H) and *OfHR3* (I) at 1 h after treatment with different concentrations of 20E. Total RNA was isolated and mRNA expression levels were determined using the quantitative real-time polymerase chain reaction. The data represent mean values of three independent samples, normalized relative to *OfRpL3* transcript levels. 0.005  $\mu$ g/50  $\mu$ l of JHA alone or 0.005  $\mu$ g/50  $\mu$ l of 20E alone was taken as the calibrator sample.



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