

# Identification and Gene Expression Analysis of the Pheromone Biosynthesis Activating Neuropeptide Receptor (PBANR) From the *Ostrinia furnacalis* (Lepidoptera: Pyralidae)

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## Abstract

Pheromonal communication is important in insect mate finding and reproduction. Identifying components of pest insect pheromone system is a first step to disrupt pest insect reproduction. In this study, we identified and cloned the pheromone biosynthesis activating neuropeptide receptor (PBANR) from the Asian corn borer, *Ostrinia furnacalis* (Guenée) (Lepidoptera: Pyralidae), which is one of the most damaging pests of corn and other crops in parts of Asia and Australia. The *O. furnacalis* PBANR (*OstfuPBANR*) gene has an ORF of 1,086 bp and encoded 362 amino acids with seven transmembrane domains and had a high sequence identity to known lepidopteran PBANRs. Expression analysis showed that *OstfuPBANR* was highly expressed in the pheromone glands compared with other tissues, consistent with other studies. Interestingly, *OstfuPBANR* was expressed higher in the larval stages compared to the pupal or adult stages, suggesting that *OstfuPBANR* may have broad functions in larva beyond adult pheromone synthesis.

**Key words:** Asian corn borer, sex pheromone, pheromone biosynthesis activating neuropeptide receptor (PBANR), pbanr gene, expression pattern

The Asian corn borer, *Ostrinia furnacalis* (Guenée) is one of the most destructive pests in Asia, Australia, and the Solomon Islands. *Ostrinia furnacalis* larvae damage corn crops by consuming new leaves and boring into the stalks and cobs, which reduce crop quality and production each year (Wang et al. 2000, Afidchao et al. 2013). To control this pest, several strategies have been developed, including chemical control (Wei and Du 2004, Liu et al. 2008) and biological control (Tran and Hassan 1986, Chen et al. 2013). Among these strategies, chemical control has been recommended as one of the most efficient methods to control this agricultural pest and prevent potential economic loss (Oerke and Dehne 2004). However, increased and repeated uses of pesticides have led to the emergence of resistant strains and have become a concern due to potential environmental pollution (He et al. 2006, Fan et al. 2016). The microbial pesticide *Bacillus thuringiensis* (Bt) has been widely used in controlling lepidopteran agricultural pests but resistant strains of these pests have been reported in the field and laboratory (Siegwart et al. 2015), including *O. furnacalis* (Xu et al. 2013).

Disrupting the pheromonal communication systems in pest insects can be a potential alternative strategy in pest management. The

use of sex pheromone is a major mode of communication in insect mate-finding, especially in Lepidoptera (Koutroumpa and Jacquin-Joly 2014). One of the most important receptor genes involved in the biosynthesis of sex pheromones is the pheromone biosynthesis activating neuropeptide receptor (PBANR) (Choi et al. 2003, Rafaeli et al. 2003).

PBANR was first identified from the pheromone glands of *Helicoverpa zea* (Choi et al. 2003). Subsequently, PBANR was cloned and identified separately from female moths of *Bombyx mori* (Hull et al. 2004) and other species (Choi et al. 2013, Jiang et al. 2018). PBAN binding to its receptor (PBANR) opens a ligand-gated Ca<sup>2+</sup> channel in gland cells to cause the influx of extracellular Ca<sup>2+</sup>, which then initiates the biosynthesis and release of the sex pheromone (Jurenka et al. 1991, Choi and Jurenka 2006). PBANRs exist as multiple variants or protein isoforms because of alternative splicing, and each of the PBANR isoforms described is different only at the C-terminus. The multiple PBANR isoforms could play different functional roles in regulating sex pheromone biosynthesis in diverse moth species (Lee et al. 2012a,b; Fodor et al. 2018). For example, in *Ostrinia nubilalis* three PBANR

isoforms were identified: PBANR-A, PBANR-B, and PBANR-C (Nusawardani et al. 2013). In addition, multiple PBANR variants (As, A, B, and C) were cloned from the PGs of several moth species (Lee et al. 2012b).

Knocking down the *pbanr* gene in moths by RNA interference has been reported to interfere with pheromone biosynthesis and leads to a decrease in mating success (Ohnishi et al. 2006, Bober and Rafaeli 2010, Lee et al. 2011). Therefore, targeting *pbanr* could be a novel method to control insect pests such as *O. furnacalis*. However, the *pbanr* gene in *O. furnacalis* has not been cloned and characterized. In this study, we identified *pbanr* from *O. furnacalis*

(*OstfuPBANR*) and investigated its expression in different tissues and developmental stages using quantitative real-time PCR (RT-PCR).

## Materials and Methods

### Insects

The *O. furnacalis* laboratory colony was originally collected from field corn in Gongzhuling city, Jilin province, China (43°47'54"N, 117°04'43"E), and reared on artificial diet (Zhou et al. 1980) in a growth chamber for three years at 26 ± 1°C with relative humidity of

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-155                               AGTCTTTGGCCCGCAGCCCCGCAGCGCTGCCGTGC
-120   GGACGCGAGTGTGACGAACCGGCAAAGTTTGGCGACTCGTGATTATTGTCTTATGTGCA
-60   CAAACTGGATTTTTGTGAAATGTTGATCGATTGGAACACCACAATTTTTATATGGTGTG
1     ATGCGCGCCCTAGCAAGGCCATGGAGTGGAAACAGCTCGTTTGACATGCTCATGGCAAAT
1     M  A P P S K A M E W N S S F D M L M A N
61   GTCACGAATGCATCCGACTCGCGGAGCTATGCTTACGCTGAGCCGGACTCCCTGGACCTT
21   V T N A S D S R S Y A Y A E P D S L D L
121  TTGGTCCCGCTCAGCGTCACTTACATCATCATCTTCGTGGCCGGTATCCTCGGGAACATC
41   L V P L S V T Y I I I F V A G I L G N I
      TM1
181  AGCACATGCGTGGTGATTGCAAGGAATCGATCCATGCACACCGCCACAACTTCTACCTC
61   S T C V V I A R N R S M H T A T N F Y L
      TM2
241  TTCAGTCTGGCCATATCGGACCTCCTACTCCTCGTGTGTGGTCTTCCGAGCGAATTCAC
81   F S L A I S D L L L L V C G L P S E F H
      TM3
301  CGAATGTGGAACCCGTCACATACCCGCTCGTGAAGCTCACTGCATAGCGCTGGGGCTC
101  R M W N P S T Y P L V E A H C I A L G L
      TM4
361  GCGTCAGAAACGTCAGCGAACCGACCGTTCTGACAATCACGGCCTTACCCTCGAGCGA
121  A S E T S A N A T V L T I T A F T V E R
421  TACATCGCCATATGTCGTCCTCATGTACATACCATGTCAAAGCTGTCGCGCGTGTC
141  Y I A I C R P F M S H T M S K L S R A V
      TM5
481  CGTTTCATCGTCGCGATATGGGTGTGTGCTCTGTGCACCGCGATCCCGCAGGCCATGCAG
161  R F I V A I W V C A L C T A I P Q A M Q
541  TTTGGCATAGTGACACAAATAGACAACGGGCAAACAATAAGTGTGTACTGTGAAGGGT
181  F G I V T Q I D N G Q T T I V C T V K G
601  CAGGGAGTGCATCAAGTGTATTTATATATCTAGTTTTATATTTTTTGTGGTGCCTATGTCT
201  Q G V H Q V F I I S S F I F F V V P M S
      TM6
661  GTGATTTGTGTGTTGTATGCGTTGATAGGGATTAAGTTGCGTACATCGCGGTTTTGCAC
221  V I C V L Y A L I G I K L R T S R V L H
721  CCGATTAAGAAGCTTTCAATGGAGAGCAGCGAGCGATGCAGCATGAGCACGCGCTACAGA
241  P I K K L S M E S S E R C S M S T R Y R
781  AGCGGCACCTCGCAGCGAAGAGTCATTAGGATGTTAGTGGCAGTAGCGCTGTCAATCTTC
261  S G T S Q R R V I R M L V A V A L S F F
      TM7
841  GTGTGCTGGGCGCCTTTCCACGTGCAGCGACTTCTCGCCATCTACGGGAAGAGCATGGAG
281  V C W A P F H V Q R L L A I Y G K S M E
901  AGGCCTTCAGAACTTTTTATCTGGTGTACATTGTGCTGACGTTTCTATCTGGGGTGTCTG
301  R P S E T F Y L V Y I V L T F L S G V L
      TM7
961  TACTTCTGTCCACCGCATCAACCCCATCCTCTACAACATCATGTGCAACAAATTCAGA
321  Y F L S T A I N P I L Y N I M S N K F R

1021  AATGCGTTCAAGGTTAGTGGTACTGTAATAATTGTAACACACTCGTATTTTATTTTACGT
341  N A F K V S G T V I I V T H S Y F I L R
1081  TTATTGTGATTACGTTTCAAACGGGTTGGAACCTTGCCCAACCTGAAGAATCGAAAGAGG
361  L L *
1141  TATAAGCGAGATAAAACCTTTCT

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**Fig. 1.** Nucleotide and amino acid sequences of *OstfuPBANR*. Nucleotide 1 is the A of ATG-translation initiation codon. Initiation codon (ATG) and stop cod (TGA) are in bold. The seven transmembrane domains are underlined and labeled TM1–TM7.

70% and photoperiod of 14:10 (L:D) h. Pupae were sexed based on the morphology of the genital pore and oviposition opening of the female and kept separate for emergence (Zhang et al. 2013).

### Extraction of Total RNA and Synthesis of First-Strand cDNA

Total RNA was first isolated from 15 pooled sex pheromone glands of 2-d-old virgin females of *O. furnacalis* during the 8–9 h of the scotophase using an Eastep Super Total RNA Extraction Kit (Code: LS1040, Promega, United States) following the manufacturer’s protocol. The cDNA was synthesized with oligo dT primer using a PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa 6210A, China).

### Identification of *pbanr*

A *O. nubilalis* *pbanr*-A sequence (GenBank: JX500421.1) was used to query a *O. furnacalis* full-length transcriptome database (made using total RNA from whole larvae, pupae and adults of *O. furnacalis* mixed at the same concentration) generated previously in our lab (Luo et al. unpublished). We obtained a putative 1318bp transcript, which we confirmed to be the *O. furnacalis pbanr* orthologue by an NCBI BLAST search. To obtain the ORF of the *O. furnacalis pbanr* gene, a specific gene fragment including predicted start codon (ATG) and stop codon (TAA) from the identified *pbanr* transcript was amplified using specific primers (forward primer 5'-AACCGGCAAAAAGTTTGGCA CTC-3', and reverse primer 5'-TCAGGTTGGCAAAGTTCCA-3') designed by NCBI Primer-BLAST online (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The PCR amplification was performed as follows: 5 min at 94°C, 30 cycles of 94°C for 30 s, 60°C for 30 s, and

72°C for 1 min, and a final extension at 72°C for 10 min. Amplified PCR products were run on 1.5% agarose gels with ethidium bromide staining under UV light. The PCR products were cloned into the pMD18-T vector (TaKaRa, China) and the ligated product was transformed into DH5α (TaKaRa, China) competent cells. Positive colonies were then identified by colony PCR and sequenced by the Sangon Biotech company (Shanghai, China).

### Sequence Alignment of *OstfuPBANR* With PBANR of Closely Related Species

The open reading frame (ORF) of *OstfuPBANR* was predicted by using ORF Finder online (<https://www.ncbi.nlm.nih.gov/orffinder/>). The amino acid sequences of *OstfuPBANR* and PBANRs from two closely related species, *O. nubilalis* and *Chilo suppressalis* were aligned by CLUSTALW using DNAMAN 8.0 (Lynnsoft, Vaudreuil, Quebec, Canada).

### Tissue-Specific and Life Stage-Specific RT-PCR of *OstfuPBANR*

To investigate the spatial and temporal expression patterns of *OstfuPBANR*, RNA was extracted from four different tissues (head, thorax, ovary and pheromone gland) of 15 two-day-old *O. furnacalis* virgin females during the 8–9 h of the scotophase as well as four whole animals from different life stages (3rd instar larvae, 5th instar larvae, pupae days 1–6 and adult days 1–6). Total RNA extractions were performed as described above. RNA quality and quantity were assessed using a NanoDrop2000 spectrophotometer (NanoDrop). Single-stranded cDNA was synthesized from 500 ng total RNA with

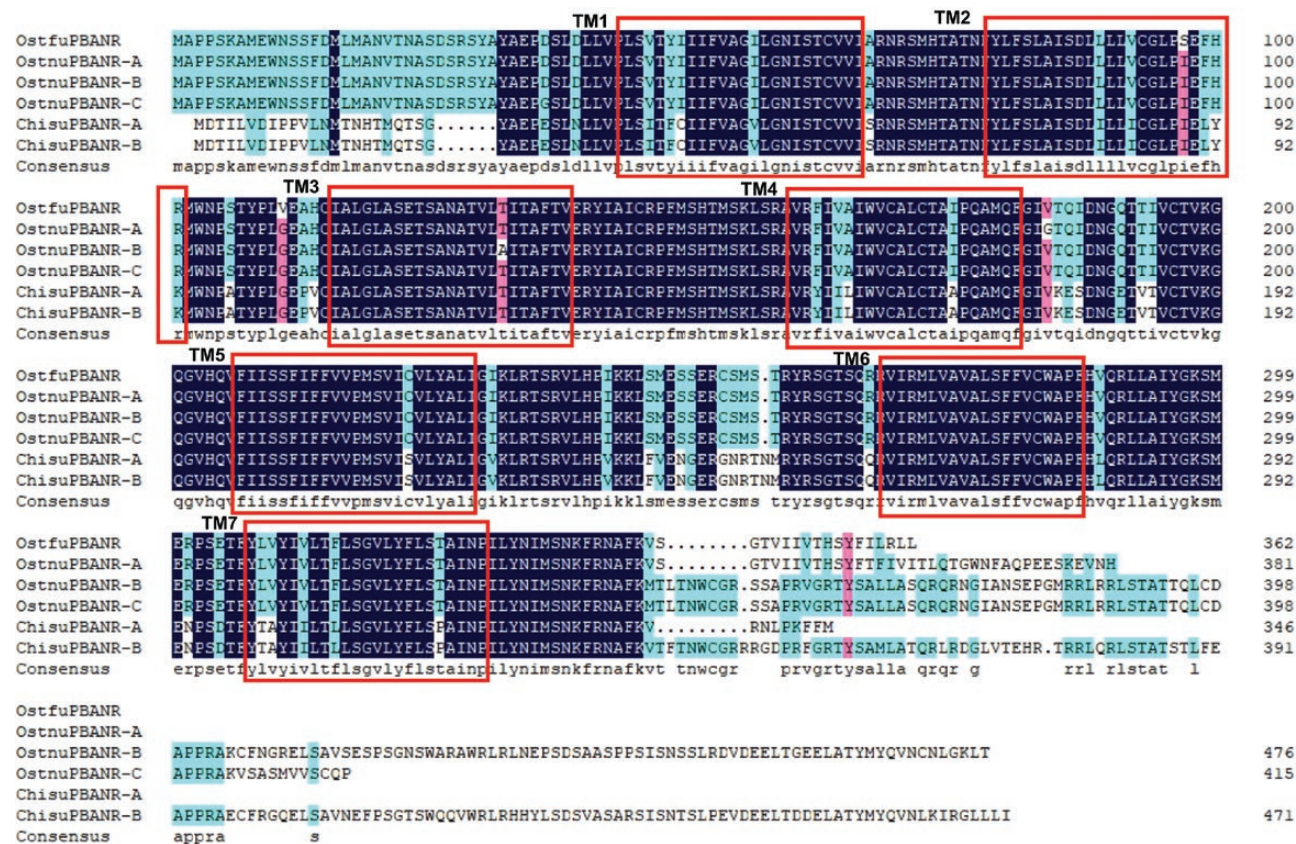


Fig. 2. Alignment of the amino acid sequence of *OstfuPBANR* with those PBANRs of family Pyralidae (Lepidoptera). The dark blue color indicated consensus amino acid residues. The transmembrane domains are indicated in red boxes and labeled TM1-TM7. GenBank accession numbers: *OstfuPBANR* (MK288169); *O. nubilalis* PBANR-A (AGL12066.1), PBANR-B (AGL12067.1) and PBANR-C (AGL12068.1); *C. suppressalis* PBANR-A (ALM88337.1) and PBANR-B (ALM88338.1).

a mixture of oligo dT and random 6 mers using the PrimeScriptRT reagent Kit with gDNA Eraser (RR047A, TaKaRa, China).

Quantitative Real-time PCR was performed on a 7500 Real-Time PCR System (ABI thermocycler) and using SYBR Premix Ex Taq II kit (RR420A, TaKaRa, China) in a 20  $\mu$ l reaction volume: 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Specific primers of Real-time PCR are used for *OstfuPBANR* (forward primer 5'-CTGTGAAGGGTCAGGGAGTG-3' and reverse primer 5'-CAAACCCGCGATGTACGC-3'). RpS3 was used as the reference gene (forward primer 5'-ATCGCGCAAGCAGAGTCTTGAGA-3' and reverse primer 5'-AACTTCATTGACTTGGCACGCTGG-3'). Primer efficiencies were 103.9 and 91.7%, respectively. Relative gene expression was calculated by the Pfaffl method due to differences in primer efficiencies (Pfaffl 2001). Each experiment consisted of three technical replicates and three biological replicates.

### Statistical Analysis

One-way analysis of variance (ANOVA) for expression levels of different tissues and different developmental stages by using the statistical software SPSS (Ver. 24.0, SPSS Inc., Chicago). Post hoc analysis was conducted using Tukey's HSD test at  $\alpha = 0.05$  if significant differences were found.

## Results

### Identification, Cloning and Sequence Analysis of *OstfuPBANR*

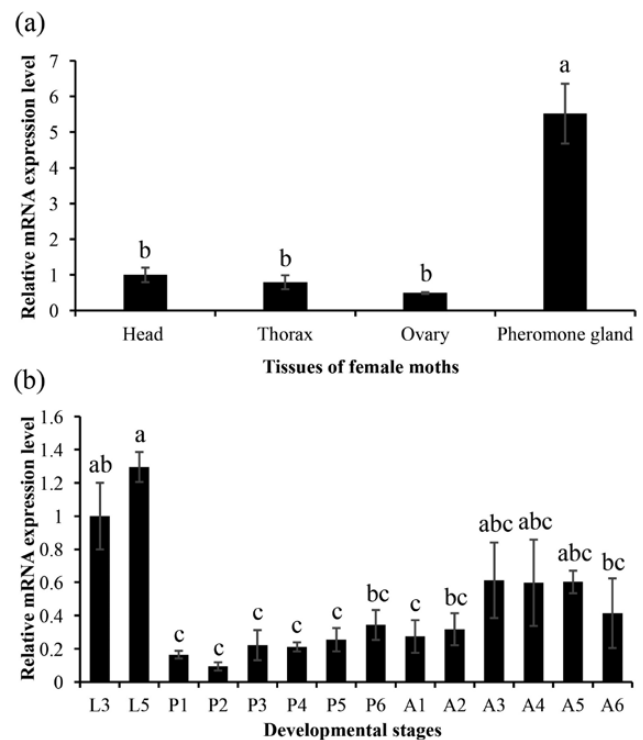
We have identified and cloned the *O. furnacalis* PBANR (*OstfuPBANR*) (GenBank: [MK288169](#)). *OstfuPBANR* has an ORF of 1,086 bp which encodes 362 amino acids (Fig. 1). Sequence analysis of *OstfuPBANR* showed seven predicted transmembrane domains (TM) similar to other G protein-coupled receptors. *OstfuPBANR* protein was blasted to the nonredundant protein database of NCBI using BLASTP. Highest hits from BLASTP was to *O. nubilalis* PBANR isoform A (99% identity), isoform B (98%), isoform C (98%), and *C. suppressalis* PBANR isoform A (83%) and isoform B (82%). CLUSTALW multiple sequence alignment of the different PBANR isoforms showed that the major difference occurs near the N-terminus and C-terminus, especially in C-terminus (Fig. 2).

### Expression Pattern of *OstfuPBANR*

To investigate the spatial expression of *OstfuPBANR*, we compared the expression of *OstfuPBANR* in four different tissues of 2-d-old *O. furnacalis* virgin females using RT-PCR. We found that expression of *OstfuPBANR* varies in these tissues ( $F_{(3,8)} = 29.084$ ;  $P = 0.004$ ) with the highest expression in the pheromone gland (Fig. 3a). RT-PCR of *OstfuPBANR* across different life stages (Fig. 3b) showed that the highest relative expression of *OstfuPBANR* occurs during larval stages, consistent with previous observations in a closely related species, *O. nubilalis* (Nusawardani et al. 2013).

## Discussion

In this study, we successfully identified and cloned the *pbanr* gene from *O. furnacalis*, and examined its expression in different life stages and different adult tissues. A 1086 bp transcript was identified which included a complete ORF, encoding a protein of 362 amino acids with seven transmembrane domains. *OstfuPBANR* has a high amino acid identity to other PBANRs of Lepidoptera insects (Jiang et al. 2018),



**Fig. 3.** mRNA expression of *OstfuPBANR* in different tissues of (a) female adult and (b) different developmental stages. L3 and L5: 3rd and 5th instar larvae; P1–P6: day 1–6 pupae; A1–A6: day 1–6 adults. Statistical analysis was performed by one-way analysis of variance followed by Tukey's HSD test ( $\alpha = 0.05$ ).

except at the N- and C-terminal ends, consistent with previous observations of other GPCR family genes. Previous studies have identified one to four isoforms of this gene in different Lepidopteran species (Lee et al. 2012b, Nusawardani et al. 2013). In the closely related species *O. nubilalis*, three PBANR isoforms were isolated (Nusawardani et al. 2013). In our experiments, we only identified a single isoform of PBANR in *O. furnacalis*. This could be due to other isoforms of PBANR in *O. furnacalis* being expressed at low levels in our transcriptomic data or it could be the case that *O. furnacalis* has only one PBANR isoform.

We also examined the expression of *OstfuPBANR* and showed that its highest expression is in the pheromone gland, similar to the expression of *pbanrs* in other lepidopterans (Cheng et al. 2010, Lee et al. 2011, Nusawardani et al. 2013, Fodor et al. 2018, Jiang et al. 2018). RT-PCR of *OstfuPBANR* from different life stages showed the highest expression of *OstfuPBANR* occurs in the larval stages. This suggests that *pbanr* may have other functions in the larval stages, such as cuticular melanization or potential regulation of pupal diapause (Zheng et al. 2007). This is consistent with a previous study in *O. nubilalis* showing that the highest expression of *pbanr* occurs in the larval stages, especially in the fat body (Nusawardani et al. 2013). Expression of *pbanr* in the fat body was also detected in the Chinese oak silkworm *A. pernyi* (Jiang et al. 2018), but not in the silk moth *B. mori* (Hull et al. 2004), suggesting the expression pattern of *pbanr* may be different amongst closely related moth species. In addition, in contrast to Nusawardani et al. who showed that *pbanr* expression is higher in the pupal stage than the adult stage in *O. nubilalis* (Nusawardani et al. 2013), we showed that *pbanr* expression in adults is higher than in pupae of *O. furnacalis*, consistent with many other lepidopterans (Cheng et al. 2010, Jiang et al. 2018).

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