Identification and characterization of the pyrokinin/pheromone biosynthesis activating neuropeptide family of G protein-coupled receptors from Ostrinia nubilalis

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Abstract
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Keywords
PBAN, diapause hormone, periviscerokinins, G protein-coupled receptors, quantitative PCR

Disciplines
Biochemistry | Entomology | Genetics

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Identification and characterization of the pyrokinin/pheromone biosynthesis activating neuropeptide family of G protein-coupled receptors from *Ostrinia nubilalis*

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Abstract

Insects have two closely related G protein-coupled receptors belonging to the pyrokinin/pheromone biosynthesis activating neuropeptide (pyrokinin/PBAN) family, one with the ligand PBAN or pyrokinin-2 and another with diapause hormone or pyrokinin-1 as a ligand. A related receptor is activated by products of the *capa* gene, periviscerokinins. Here we characterized the PBAN receptor and the diapause hormone receptor from the European corn borer, *Ostrinia nubilalis*. We also identified a partial sequence for the periviscerokinin receptor. Quantitative PCR of mRNA for all three receptors indicated differential expression in various life stages and tissues. All three splice variants of the PBAN receptor were identified with all variants found in pheromone gland tissue. Immunohistochemistry of V5 tags of expressed receptors indicated that all three variants and the diapause hormone receptor were expressed at similar levels in *Spodoptera frugiperda* 9 (Sf9) cells. However, the A-and B-variants were not active in our functional assay, which confirms studies from other moths. Functional expression of the C-variant indicated that it has a 44 nM half effective concentration for activation by PBAN. The diapause hormone receptor was activated by diapause hormone with a 150 nM half effective concentration.

Keywords: PBAN, diapause hormone, periviscerokinins, G protein-coupled receptors, quantitative PCR.

Introduction

Neuropeptides are central to the coordination of animal physiology by acting as neurotransmitters, neuromodulators or as neurohormones. One major family of neuropeptides includes the pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) of insects and neuromedin U in vertebrates (Jurenka & Nusawardani, 2011). This family of neuropeptides is grouped together based on the conserved C-terminal sequence of FXPRLamide that is required for activity (Rafaeli & Jurenka, 2003). The PK/PBAN neuropeptides are ancient conserved signalling molecules that were most likely first utilized to modulate muscle contraction as a peripheral action and to coordinate feeding behaviour and/or energy homeostasis as a central action (Bader et al., 2007; Saideman et al., 2007). Subsequently the family of peptides and receptors evolved into regulating a variety of physiological functions in various animals. In insects the known functions include stimulation of muscle contraction in moths (PBAN), stimulation of muscle contraction (PK), induction of embryonic diapause in the silkworm moth [diapause hormone (DH)], induction of ecdysteroidogenesis in *Bombyx mori*, acceleration of puparium formation in higher flies, stimulation of cuticular melanization in moth larvae and modulation of pupal diapause development in heliothine moths (Rafaeli & Jurenka, 2003; Rafaeli, 2009).

In most insects, two genes encode for several PK/PBAN-like peptides, which include PBAN (aka PK2), DH (aka PK1) and periviscerokinins (PVK). Receptors for all three peptides have been identified in insects and are very closely related with regard to primary and secondary structures (Jurenka & Nusawardani, 2011). In the present study we identified the PK/PBAN family of G protein-coupled receptors (GPCRs) in the European corn borer...
moth, *Ostrinia nubilalis*. Previous research has demonstrated the role of PBAN in stimulation of pheromone biosynthesis in *O. nubilalis* and localization of PBAN-like neuropeptides in the central nervous system of this moth (Ma & Roelofs, 1995a, 1995b). Previous studies have identified the PBAN (Hull et al., 2004) and DH (Homma et al., 2006) receptors in *B. mori*. The PVK-receptor (PVK-R) has not been functionally characterized in *B. mori* but its expression pattern was determined in a study with other neuropeptide receptors (Yamanaka et al., 2008). The PBAN-receptor (PBAN-R) gene sequence has been identified in a about a dozen lepidopteran species but the DH-receptor (DH-R) has only been characterized in *B. mori* (Homma et al., 2006) and the gene sequence identified in the white-spotted tussock moth, *Orgyia thyellina* (AB283041). The present report describes the characterization of all three closely related receptors in *O. nubilalis*, which is a major pest of maize.

**Results**

**Cloning of receptors**

A PCR-based cloning strategy using degenerate primers designed against highly conserved sequences of known receptors was utilized in this study. Full-length cDNA clones were obtained for PBAN-R and DH-R and used to obtain full open reading frame (ORF) sequences. Only a partial sequence was obtained for the PVK-R. Fig. 1 shows the complete deduced amino acid sequences for these clones. A BLAST search of GenBank using the deduced amino acid sequences revealed similarities with known invertebrate and vertebrate GPCRs and indicated that we have identified three PBAN-R variants, the DH-R and a partial sequence of the PVK-R (or CAPA-R). These receptors were aligned with the corresponding receptors from *B. mori* because it is currently the only lepidopteran from which all six receptors have been identified (Fig. 1). Comparison of the three PBAN-R variants indicates about 76% conserved amino acid sequence between the two species. The differences amongst the three PBAN-R variants are similar to that found in other Lepidoptera (Lee et al., 2012). The A variant is the shortest followed by the C and B variants. Comparison of the DH-R indicates a 67% conserved amino acid sequence. The partial *O. nubilalis* PVK-R has an 88% conserved amino acid sequence with *B. mori* PVK-RA25. As with other insect GPCRs in this receptor family, the transmembrane domains have a highly conserved amino acid sequence.

These alignments, along with related GPCRs from Lepidoptera, were compared phylogenetically using the neighbour-joining method (Fig. 2). The PBAN-Rs all form a branch with the Noctuidae receptors in one branch and the *B. mori* and *Manduca sexta* receptors forming another branch. The PBAN-Rs from *O. nubilalis*, *Plutella xylostella* and *Danaus plexippus* are basal. At this time fewer lepidopteran sequences are known for DH-R and PVK-R but these receptors form their own branches with the PVK-R being basal. Currently a DH-R or PVK-R have not been identified from a noctuid, although partial sequences have been obtained from pheromone glands and haemocytes, respectively, of *Heliothis virescens* (Shelby & Popham, 2009; Vogel et al., 2010).

**Quantitative real-time reverse transcriptase PCR (qRT-PCR)**

The identified sequences were used to conduct qRT-PCR of various life stages and tissues of *O. nubilalis*. Gene expression of all three receptors followed a similar pattern in the life stages examined. Relatively high expression was observed in the embryonic, first instar and pupal tissues, and in both adult sexes (Fig. 3). These expression levels were different from those of other gene products conducted with the same mRNA extracts, indicating that the profiles are reliable indicators of mRNA abundance (Kroemer et al., 2011). Various tissues from fourth and fifth larval instars were also analysed and we found that the DH-R was expressed at relatively higher levels in all tissues except the haemocytes, Malpighian tubules and nerve cord (Figs 4, 5). The PVK-R was found at the highest levels in the midgut and Malpighian tubules of fifth instars. However, the levels of the PVK-R were relatively low, which most likely contributed to the difficulty in obtaining a full-length sequence. PBAN-R was detected at the highest level in fat body of fourth instars and the midgut and heads of fifth instars (Figs 4, 5).

In adults, the PVK-R was not found in either the pheromone glands of females or the hair-pencil-aedeagus complex of males (data not shown). As expected, the PBAN-Rs were found in pheromone glands of young adult females (Fig. 6), which corresponds to when pheromone production is initiated in *O. nubilalis* (Ma & Roelofs, 1995a). In addition the DH-R was also found at relatively high levels in pheromone glands of young females. Interestingly, both receptors were also expressed at high levels in the male complexes at 72 h postemergence (Fig. 6). Amongst the three variants of PBAN-R, the expression of the A variant was detected at a similar level for the female and male glands (Fig. 7) and the expression of the B/C variants were higher in the pheromone glands of females at 24 h postemergence.

The immunocytochemistry of stably expressed receptors indicated that the PBAN-R variants and DH-R were expressed at similar levels in the transfected *Spodoptera frugiperda* 9 (Sf9) cells (Fig. 8). These results indicate that under these conditions all receptors were produced in a similar manner in the transfected Sf9 cells.
We utilized a previously described calcium assay to determine functional activity of expressed receptors with the exception that a fluorescence plate reader was used to detect changes in fluorescence intensity (Choi et al., 2003). As shown in Table 1, the DH-R was activated by *Helicoverpa zea* DH and not by *Helic. zea* PBAN. Of the

Figure 1. Alignment of *Ostrinia nubilalis* (Ostnub) receptors (R) for pheromone biosynthesis activating neuropeptide (PBAN; A, B and C variants), diapause hormone (DH) and periviscerokinins (PVK) with homologous receptors from *Bombyx mori* (Bommor). Identical amino acids are highlighted in dark grey and conserved amino acids in light grey. Note that OstnubPVK-R is a partial sequence. The predicted transmembrane domains (TM) are over-lined with =. Dashed lines indicate spaces to optimize alignment.

**Functional expression assay**

We utilized a previously described calcium assay to determine functional activity of expressed receptors with the exception that a fluorescence plate reader was used to detect changes in fluorescence intensity (Choi et al., 2003). As shown in Table 1, the DH-R was activated by *Helicoverpa zeas* DH and not by *Heli. zeas* PBAN. Of the
PBAN-R variants only PBAN-RC was highly active with no activity observed for PBAN-RA and B. PBAN activated PBAN-RC at lower concentrations than did DH.

Discussion

The O. nubilalis DH-R has a protein sequence that is similar to those identified from other Lepidoptera and from other insects (Jurenka & Nusawardani, 2011). Activation of DH-R by the DH peptide had a low half maximum effective ligand concentration (EC\textsubscript{50}) value and was not activated by PBAN. Similar results have been observed using different assay systems in other insects including B. mori (Homma et al., 2006), Anopheles gambiae (Olsen et al., 2007), Drosophila melanogaster (Cazzamali et al., 2005) and Rhodnius prolixus (Paluzzi & O’Donnell, 2012). By contrast, the O. nubilalis PBAN-RC was activated not only with a low concentration of PBAN but also by DH, although at higher concentrations. The differential activation of PBAN-R by PBAN and DH has also been observed in other insects including Helic. zea (Choi et al., 2003), Helio. virescens (Kim et al., 2008), A. gambiae (Olsen et al., 2007) and D. melanogaster (Park et al., 2002). These results indicate that the DH-R is more discriminatory in peptide activation than the PBAN-R in these insects. The three-dimensional structure of these receptors is important to allow certain peptides into the binding pocket. This is illustrated by a study in which the extracellular domains were exchanged between the Helic. zea PBAN-RA and D. melanogaster PK1-R (DH-R), with the results indicating that the third extracellular domain is important for ligand recognition (Choi et al., 2007).

Quantitative expression analysis indicated that the mRNA for all three receptors (PBAN-R, DH-R and PVK-R) follows a similar developmental profile (Fig. 3). This suggests that production of mRNA for all receptors is most

![Figure 2](phylogenetic_relationships.png)

**Figure 2.** Phylogenetic relationships of Lepidoptera periviscerokinins (PVK), diapause hormone (DH) and pheromone biosynthesis activating neuropeptide (PBAN) receptors (R) based on the neighbour-joining method. The Drosophila melanogaster ecdysis-triggering hormone receptor variant A (DromelETHrA) was used as the outgroup. The bootstrap percentage values (1000 replicates) are shown next to the branches. The evolutionary distance is represented by the scale bar and the units (0.05) are the number of amino acid substitutions per site. The GenBank accession numbers are listed next to the species and receptor abbreviations. Species abbreviations are the first three letters of genus and species names combined. Species: Heliothis virescens, Helicoverpa armigera, Heliothis peltigera, Helicoverpa zea, Spodoptera littoralis, Spodoptera exigua, Mythimna separata, Bombyx mori, Manduca sexta, Danaus plexippus, Plutella xylostella, Orgyia thyellina.

![Figure 3](normalized_expression.png)

**Figure 3.** Normalized expression of periviscerokinin receptor (PVK-R), diapause hormone receptor (DH-R) and pheromone biosynthesis activating neuropeptide receptor (PBAN-R) mRNA to an internal control gene ribosomal protein s3 at different Ostrinia nubilalis life stages using quantitative real-time reverse transcriptase PCR. E, embryonic egg masses; L1, first instars; L2, second instars; L3, third instars; L4, fourth instars; L5, fifth instars; P, pupa; F, adult females; M, adult males. Each point represents the mean and standard error of values obtained from three biological replicates. Columns with the same letter are not significantly different at the 5% significance level as determined by one-way ANOVA followed by the Tukey–Kramer honestly significant difference means separation test.
likely under similar regulatory mechanisms during development. However, relative gene expression varied in a tissue-specific manner. The PVK-R was found at the highest levels in the midgut. This is consistent with the activity of PVK peptides in regulating water balance in other insects (Pollock et al., 2004; Paluzzi et al., 2010; Terhzaz et al., 2012). In a study using B. mori in which the gene expression of neuropeptide GPCRs was determined in various tissues of fourth and fifth larval instars, the PVK-R A25 was found expressed in only the brain and muscle tissue (Yamanaka et al., 2008). The PVK-R A27 was not expressed in any of the examined tissues. In R. prolixus the PVK peptides were found to have antidiuretic activity and the PVK-R had the highest expression levels in the Malpighian tubules and anterior midgut, known sites of antidiuretic hormone activity in this insect (Paluzzi et al., 2010). In M. sexta, a PVK peptide (aka Cap2b) did not have any influence on fluid secretion in Malpighian tubules (Skaer et al., 2002). It remains to be determined if the PVK peptides play a role in maintaining water balance in Lepidoptera through the midgut as indicated by the expression of the PVK-R in the midgut of O. nubilalis. The PVK peptide does have cardioacceleratory activity in M. sexta, indicating that the heart will have a PVK-R (Huesmann et al., 1995). In our study, mRNA for PVK-R in the heart may have been present in the carcasses of fourth and fifth instar larvae, although to determine if this was the case heart tissue would have needed to be dissected and analysed directly.

The DH-R was found expressed at the highest levels in the head, fat body and midgut of O. nubilalis larvae. It was also found in pheromone glands of adult females and hair-pencil-aedeagus complexes of adult males. In B. mori, DH-R binds the DH peptide to induce embryonic diapause in the ovaries. The expression of DH-R in ovaries of adult B. mori females confirms this role (Homma et al., 2006). B. mori DH-R mRNA was also found in low levels in various tissues and at higher levels in the prothoracic gland of the fifth instar and pheromone glands of adults (Watanabe et al., 2007). Furthermore, it was demonstrated that the DH peptide could stimulate ecdysteroid production by the prothoracic glands in B. mori (Watanabe et al., 2007). In a study by Yamanaka et al. (2008), the DH-R was found to be highly expressed in the silk glands and fat body of fourth and fifth instars. Expression of the DH-R in various tissues indicates that

![Graphical representation of gene expression](image1)

**Figure 4.** Normalized expression of periviscerokinin receptor (PVK-R), diapause hormone receptor (DH-R) and pheromone biosynthesis activating neuropeptide receptor (PBAN-R) mRNA to ribosomal protein s3 in the indicated tissues of Ostrinia nubilalis fourth (4th) and fifth (5th) instar larvae using quantitative real-time reverse transcriptase PCR. Each point represents the mean and standard error of values obtained from three biological replicates. Columns with the same letter are not significantly different at the 5% significance level as determined by one-way ANOVA followed by the Tukey–Kramer honestly significant difference means separation test.

![Graphical representation of gene expression](image2)

**Figure 5.** Normalized expression of periviscerokinin receptor (PVK-R), diapause hormone receptor (DH-R) and pheromone biosynthesis activating neuropeptide receptor (PBAN-R) mRNA to ribosomal protein s3 in various tissues of Ostrinia nubilalis fifth instar larvae using quantitative real-time reverse transcriptase PCR. MG, midgut; MT, Malpighian tubule; HD, head; NC, nerve cord. Each point represents the mean and standard error of values obtained from three biological replicates. Columns with the same letter are not significantly different at the 5% significance level as determined by one-way ANOVA followed by the Tukey–Kramer honestly significant difference means separation test.

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the DH peptide signalling system may serve a variety of functions in Lepidoptera.

PBAN-R was found to be expressed in pheromone glands of adult females and at lower levels in hair-pencil-aedeagus complexes of adult males. Of the three variants, B and C were found at higher levels. The B and C variants were also found to be expressed at higher levels than the A variant in other moths (Lee et al., 2012). These results, coupled with the finding that the *O. nubilalis* PBAN-RC variant had the highest functional activity in our expression system, indicates that this variant is the active receptor in pheromone glands. This finding corroborates reports in which the C variant was the most active in various moths (Kim et al., 2008; Lee et al., 2012). However, the A variant was also active as demonstrated in *Helic.zea* (Choi et al., 2003) and Spodoptera littoralis (Zheng et al., 2007). Detection of activity may be coupled to the assay system employed. In the two studies in which the A variant was found to be active, a fluorescent microscope was employed to detect individual cells that responded to ligand challenge. In other studies, including the present study, a fluorescence or luminosity reader was utilized that detects the signal from the whole well of a plate. These differences could account for the sensitivity of the assays and the variation found amongst studies in determination of activity.

In addition to being expressed in pheromone glands, PBAN-R was also found to be expressed in the male hair-pencil-aedeagus complex of *O. nubilalis*. These

![Figure 6. Normalized expression of diapause hormone receptor (DH-R) and pheromone biosynthesis activating neuropeptide receptor (PBAN-R) mRNA to ribosomal protein s3 in adult female pheromone glands (PG) and hair-pencil-aedeagus complexes of males (MC) using quantitative real-time reverse transcriptase PCR. PG1, PG2, PG3, from females 1, 2 and 3 days postemergence, respectively. MC1, MC2, MC3, from males 1, 2 and 3 days postemergence, respectively. Each point represents the mean and standard error of values obtained from three biological replicates. Columns with the same letter are not significantly different at the 5% significance level as determined by one-way ANOVA followed by the Tukey–Kramer honestly significant difference means separation test.](image)

![Figure 7. Normalized expression of pheromone biosynthesis activating neuropeptide receptor variants mRNA to ribosomal protein s3 in adult female pheromone glands (PG) and male hair-pencil-aedeagus complexes (MC) using quantitative real-time reverse transcriptase PCR. PG1, PG2, PG3, from females 1, 2 and 3 days postemergence, respectively. MC1, MC2, MC3, from males 1, 2 and 3 days postemergence, respectively. Each point represents the mean and standard error of values obtained from three biological replicates. Columns with the same letter are not significantly different at the 5% significance level as determined by one-way ANOVA followed by the Tukey–Kramer honestly significant difference means separation test.](image)
results are consistent with the recent findings that PBAN can stimulate the production of male hair-pencil components in *Helicoverpa armigera* (Bober & Rafaeli, 2010) and that PBAN-R is found in the hair-pencil-aedeagus complex of adult males (Rafaeli et al., 2007). Expression of PBAN-R in various tissues, including the nerve cord and brain, has also been found in *B. mori* and *Helic. armigera* (Hull et al., 2004; Yamanaka et al., 2008). Expression of PBAN-R in various tissues indicates that the PBAN signalling system may serve a variety of functions in Lepidoptera.

In conclusion, we have identified three GPCRs from the moth *O. nubilalis*. The PVK-R as a partial sequence was used to conduct qRT-PCR of life stages and tissues. The ORFs of all three variants of the PBAN-R were identified with the C-variant being the most active and most abundantly expressed in pheromone gland cells of females and the hair-pencil-aedeagus complex of males. Likewise, the DH-R ORF was expressed in pheromone glands of females and the hair-pencil-aedeagus complex of males, indicating possible involvement of DH in regulating pheromone production. Both receptors are also expressed in other tissues, indicating possible roles in regulating other physiology. These results point to the general pleiotropic nature of the PK/PBAN signalling system in insects. The PVK-R was primarily expressed in midgut tissue, indicating a potential role in regulating water balance.

**Experimental procedures**

**Insects**

*Ostrinia nubilalis* were obtained from a laboratory at the United States Department of Agriculture – Agricultural Research Service, Corn Insects and Crop Genetics Research Unit, Ames, IA, USA. Life-cycle and tissue samples were collected as described in Kroemer et al. (2011). Whole insects in different life stages were collected as: whole embryonic egg masses, pre-emergent blackhead egg masses, second, third, fourth and fifth instars, pupae, and male and female adults. Haemocytes were collected from fourth and fifth instar larvae by cutting the first proleg, bleeding into ice-cold phosphate-buffered saline (PBS) and washing three times in ice-cold PBS. Fat bodies, Malpighian tubules, nerve cords, heads, digestive tracts and carcasses from fourth and fifth instar larvae were placed separately in 1.5 ml tubes containing 500 μl ice-cold PBS. Tissues were then washed three times with ice-cold PBS. Pheromone glands from adult females and hair-pencil-aedeagus complexes from adult males were collected 1, 2 or 3 days after adult emergence and the glands were washed three times with ice-cold PBS.

**RNA extraction and 5′ and 3′ rapid amplification of cDNA ends (5′ and 3′ RACE) amplification**

Life-cycle and tissue samples were collected and total RNA was extracted in 1 ml of Trizol according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). cDNA synthesis reactions were performed on 2 μg total RNA from all pools of *O. nubilalis* life-stage and tissue samples using Moloney murine leukemia

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>PBAN-RA</th>
<th>PBAN-RB</th>
<th>PBAN-RC</th>
<th>DH-R</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Helic. zeae</em> PBAN</td>
<td>LSDDMPATPADOEMYROPEOIDSRTKYFSPRLa</td>
<td>NA</td>
<td>NA</td>
<td>44 nM</td>
<td>NA</td>
</tr>
<tr>
<td><em>Helic. zeae</em> DH</td>
<td>NDVKDGAASGAHSDRLGLWGFSPRLa</td>
<td>NA</td>
<td>NA</td>
<td>191 nM</td>
<td>150 nM</td>
</tr>
</tbody>
</table>

NA indicates no activity at a 1 μM concentration of ligand.

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virus reverse transcriptase according to the manufacturer’s protocol (Promega Corporation, Madison, WI, USA). cDNA was synthesized with 3 μg total RNA from pheromone gland tissues and fifth instar heads using the SMART IV cDNA Library Construction Kit (Clontech, Mountain View, CA, USA) with CDS III (5′-ATT CTG GAG GCC GAG GCG GCC GATC-3′) and SMART IV (5′-AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ACG GCC GGG-3′) synthesis primers.

Nested 5′ or 3′ RACE amplification reactions were performed by using gene-specific forward primer with the CDS III primer and the CDS III 3′ primer (5′-AGG CAG ACG GCG ACA T-3′) or gene-specific reverse primers with the SMART IV primer and the SMART IV 5′ primer (5′-AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ACG GCC GGG-3′) synthesis primers. PCR products for RACE were performed using Platinum Taq High Fidelity DNA polymerase (Invitrogen, Carlsbad) according to the manufacturer’s protocol. Synthesized cDNA from female pheromone glands and fifth instar heads were amplified with a sense primer of 5′-ATG CAY ACI ACI AAY TWY TAY YTI TT-3′ and an antisense primer of 5′-OKY TGY GCR TGR AAI GGY ICIC CCA-3′ as described in Homma et al. (2006). In the case of the PKV-R, a product was sequenced from fifth instar heads but we could not obtain the 5′ and 3′ ends. PCR products from pheromone glands were sequenced and used to design gene-specific primers to obtain 5′ and 3′ ends of the PBAN-R and DH-R cDNA. The 5′ RACE reaction using cDNA from pheromone glands was performed by using the gene-specific PBAN-R primer 5′-CGA GTG GGT ATG TGG ACG GG-3′ and the SMART IV primer. The 5′ PCR nested reaction was then performed by using gene-specific PBAN-R nested primer 5′-ACA TAC GAG GAG TAG GAG GTC-3′ and the SMART IV 5′ primer. To obtain the ORF of the DH-R from pheromone glands, the 5′ RACE reaction was performed by using the DH-R forward primer 5′-TCT GAG CAT CAC ACA GAA CGC TGG-3′ and the SMART IV primer, whereas the nested PCR reaction was performed by using the DH-R nested primer 5′-GCA TTC CGC TCA CAA GCA GAA G-3′ and the SMART IV 5′ primer. 3′ RACE was performed by using the PBAN-R forward primer 5′-GGT TTG TAT CCG ATG TTA GGG-3′ and the CDS III primer. The nested PCR reaction was then used to obtain the 3′ end of the PBAN-R gene by using the PBAN-R nested primer 5′-TAG GAT GTT AGT GGC AGT AGC-3′ and the CDS III 3′ primer. To obtain the 3′ end of DH-R, the DH-R front primer 5′-ACA ATG ACG AGC AAG GAG GATC-3′ and the CDS III primer were used to obtain the 3′ end of DH-R, and then the DH-R nested primer 5′-AGT GGT GAA GAT GTT AGT GGC-3′ and the CDS III 3′ primer were used for the nested PCR reaction. The nucleotide sequences for the O. nubilalis PKV-R, DH-R and PBAN-R variants A, B and C reported in this paper have been submitted to GenBank with accession numbers JX500424, JX500425, JX500421, JX500422 and JX500423, respectively. Deduced protein sequences were aligned using CLUSTAL W (Thompson et al., 1994) and the JalView alignment editor (Waterhouse et al., 2009). Phylogenetic tree analysis was performed by using MEGA 5.05. The neighbour-joining method was used and the bootstrap tests were performed with 1000 iterations (Tamura et al., 2007).

qRT-PCR

cDNA pools were subjected to 45 rounds of PCR in the presence of SYBR green dye (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. Reactions were performed in three biological replicates for each life stage and tissue sample. Mean starting quantities were calculated and normalized to O. nubilalis ribosomal protein s3 (rps3) by using the primers 5′-ATC GCG CAA GCA GAG TCT TTG AGA-3′ and 5′-AAC TTC ATT GAC TGG TGG CAC GCG TGG-3′, and plotted for each cDNA sample to determine the mean relative starting quantities. All qRT-PCR reactions were performed using the following programme: initial incubation of 95 °C for 3 min was followed by 45 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s. PCR efficiency and correlation coefficient (R2) values were taken into consideration prior to determining the normalized expression. Primers used to detect the DH-R mRNA were 5′-CAG CAA CGC AAG TGT TCT GAC CAT-3′ and 5′-ACC GAA CAT GAG TTG AGG TAA-3′. Forward and reverse primers, 5′-TTC ATC GTC GCC ATG AGA GTG TGT-3′ and 5′-AAA CAC TAC ATG TAC TCC CTG ACC-3′ were used to detect mRNA of all PBAN-R variants. To detect PKV-R mRNA, forward primer 5′-TAC CCA CCG AAT TCC GGC AAC ATA-3′ and reverse primer 5′-TGT CCA CTT GAT ATG CAG TCC CAT-3′ were used. Primers 5′-TGC GTT CAA GGT TAG TGG TAC TGT-3′ and 5′-ACC TCT TCC GAT TCG TCA TCT GGG TGC GC-3′ were used to detect PBAN-R variant A mRNA. Primers 5′-ACT GCA ACC TAG GCA AAC TCA CCT-3′ and 5′-ACC CGT AAA GCG AGA ACC ACT GAA-3′ were used to detect PBAN-R variants B and C mRNA. Real-time PCR reactions were performed on a Bio-Rad MyIQ Single Color Real-time PCR Thermalcycler. Melt curve analyses verified that single products were amplified for each primer set and gene identities of qPCR products were verified by sequencing. PCR primer efficiencies for each gene-specific primer set were verified to be within 5% of the rps3 by comparative standard curve analyses run concurrently with unknowns on each plate. Values for unknowns were normalized to rps3 quantities and analysed by the comparative threshold cycle method. JMP Pro 10 software (SAS Institute Inc., Cary, NC, USA) was used to analyse the normalized expression data of the different stages and tissues. One-way ANOVA tests followed by Tukey–Kramer honestly significant difference mean separation tests were performed to find out whether means were significantly different between treatments.

Receptor characterization

Spodoptera frugiperda 9 insect cells (Invitrogen, Carlsbad) were maintained in SF-900 II serum free medium (Invitrogen, Carlsbad) at 28 °C. cDNA containing the ORFs of O. nubilalis DH-R and the three variants of PBAN-R were cloned into plBl/V5-His (Invitrogen, Carlsbad) by using EcoRI (5′) and SadI (3′) restriction sites. Transfections of the recombinant plBl/V5-His vectors expressing the PBAN-R variants and DH-R were performed according to the Cellfectin II (Invitrogen, Carlsbad) manufacturer’s instructions. Selection for stably transformed cells was performed with 50 μg/ml blasticidin–HCl (Fisher Scientific, Fair Lawn, NJ, USA) for 2 weeks and blasticidin-resistant cells were maintained in SF-900 II medium with 10 μg/ml blasticidin–HCl.

Immunocytochemistry of recombinant expressing SF9 cells was used to determine if receptors were expressed at similar levels. Stably transformed cells expressing DH-R and PBAN-R containing the VS epitope or untransformed cells were allowed to attach on cover-slips. Cells were fixed in 3.7% formaldehyde–PBS for 20 min, permeabilized with 0.2% Triton X-100 plus PBS (PT) for 30 min at room temperature (RT), and blocked for 1 h with PT plus 0.1% bovine serum albumin. The cells were incubated
overnight at 4 °C in a 1:100 dilution of primary rabbit anti-V5 antibody (Sigma-Aldrich, St. Louis, MO, USA) in PT. Cells were washed three times with ice-cold PT and incubated for 45 min at 37 °C with a 1:500 dilution of goat anti-rabbit immunoglobulin-G–Alexa Flour 488 conjugated secondary antibody (Invitrogen, Camarillo, CA, USA). Cells were then washed in PT and counterstained with 300 nM 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Invitrogen, Eugene, OR, USA) in PT for 15 min at RT. DAPI-stained cells were washed three times with ice-cold PT and mounted to microscope slides for visualization using a Nikon Eclipse 50i epifluorescent microscope (Nikon, Melville, NY, USA).

To functionally characterize the receptors expressed in stably transformed Sf9 cells, a calcium assay was used. The preparation of cells, peptides and Fluo-4AM (Molecular Probes, Invitrogen, Germany) equipped with 485 nm excitation and 520 nm emission filters. Fluorescence measurements from each well were taken using a NovoStar plate reader (BMG Labtech, Ortenberg, Germany) and analyzed using a Nikon Eclipse 50i epifluorescent microscope (Nikon, Melville, NY, USA).

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References


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