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Transcript analysis and comparative evaluation of shaker and slowmo gene homologues from the European corn borer, Ostrinia nubilalis

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Keywords
insect behaviour, gene expression, slowmo, shaker, mRNA splicing

Disciplines
Entomology

Comments
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Transcript analysis and comparative evaluation of *shaker* and *slowmo* gene homologues from the European corn borer, *Ostrinia nubilalis*

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Abstract

The movement and dispersal of larval Lepidoptera impact their survival and distribution within the natural landscape. Homologues of the *Drosophila* behaviour-linked genes *shaker* (*shkr*) and *slowmo* (*slmo*) were identified from *Ostrinia nubilalis* (Lepidoptera: Crambidae). *Onshkr* was isolated as a 1610-nucleotide (nt) constitutively expressed transcript encoding a membrane-localized 469-amino-acid (aa) protein with a conserved tetramerization domain and the six-domain architecture necessary for the molecule to fold into an active K⁺ channel. Three expressed splice variants of 682, 970 and 1604 nt were identified for the *Onslmo* gene, and encode predicted 141 and 228 aa proteins with a conserved protein of relevant evolutionary and lymphoid interest (PRELI) domain that may function in mitochondrial protein sorting and perinuclear protein localization. *Onshkr* and *Onslmo* protein sequences aligned within monophyletic lepidopteran groups.

Keywords: insect behaviour, gene expression, slowmo, shaker, mRNA splicing.

Introduction

The manner in which agricultural pests behave in the field can have widespread impacts on the methods used to manage them, and on the durability of control measures being utilized or under evaluation for a particular species. Lepidopteran larvae feed upon vegetative and fruit-bearing portions of crop plants, and can cause economically significant levels of damage that also contribute to human food shortages worldwide (Mason *et al.*, 1996; Hellmich *et al.*, 2008). With the introduction of transgenic *Bacillus thuringiensis* (Bt) toxin expressing crops in the late 1990s, the impact of these pests, particularly *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae), has been reduced dramatically, leading to sustainable control of pest populations in recent years (Estruch *et al.*, 1997; Ferry *et al.*, 2006; Hutchison *et al.*, 2010). However, the continued use of transgenic crops and Bt biopesticides has led to heightened concern that pest resistance to Bt technologies may develop through behavioural or physiological means.

Larval movement is an important factor governing the dispersal and distribution of *O. nubilalis* in all maize systems (Davis & Onstad, 2000; Goldstein *et al.*, 2010). Current and proposed Bt high-dose/refuge strategies rely on the assumption that larvae do not move between Bt and non-Bt plants, and that high-dose exposure to transgenic plants will kill all heterozygous resistant alleles present in the population (Andow & Hutchison, 1998; Gould, 1998; Glaser & Matten, 2003; Tyutyunov *et al.*, 2008). Early concerns arose with mixed seed versus block refuge strategies for single toxin transgenic crops because of the potential for larval movement and the ingestion of sublethal amounts of Bt toxin (Mallet & Porter, 1992; Tabashnik, 1994; Onstad & Gould, 1998; Davis & Onstad, 2000). However, the use of multiple toxin (pyramided or stacked) traits within the maize tissue further reduces the likelihood of survival of resistant and heterozygous individuals (EPA, 2010).

Our understanding of lepidopteran pest behaviour at the molecular level is rudimentary, and is currently inadequate to evaluate field populations with regard to the changes in pest behaviour that may be associated with the development of behavioural resistance to transgenic crops. *O. nubilalis* larvae tend to be genetically predisposed to disperse or remain on host plant tissue following egg...
emergence (Davis & Onstad, 2000; S. E. Moser, unpubl. data), and neonates can detect and move away from toxic Bt maize tissues within an hour of egg eclosion (Goldstein et al., 2010). Similarly, Heliothis virescens larvae tend to move more frequently amongst plants within fields that contain both Bt and non-Bt cotton plants (mixtures) compared to homogenous stands (Parker & Luttrell, 1999).

The inherent ability of larvae to move away from transgenic tissues and/or ingest sublethal amounts of toxin may present valid concerns for the sustainability of current and proposed Bt refuge strategies (Davis & Onstad, 2000). If transgenic crop refuge models are to be adequately assessed as sustainable and reliable insect resistance management (IRM) practices, we must first further our understanding of pest behaviour, and how agricultural dynamics may be impacting the evolution of behavioural adaptations in economically relevant lepidopteran pests. Research from model insect systems indicate that locomotory traits may be genetically determined, and can be affected by mutation of the potassium (K\(^+\)) channel gene, shaker (shkr), and the mitochondrial product of the gene, slowmo (slmo). We have initiated novel investigations aimed at uncovering, validating and understanding the genetic elements governing larval dispersal and movement behaviour in O. nubilalis and other pest Lepidoptera. In this study, we describe the isolation, transcriptional analysis and protein localization for two behavioural gene homologues (Onshkr and Onsimo) in O. nubilalis.

In Drosophila melanogaster, shaker potassium channels and mitochondrial slowmo proteins play essential roles in modulating larval locomotion and behavioural phenotypes. D. melanogaster shaker mutants exhibited aberrant larval locomotion and physiology induced by disruption of nerve firing associated with movement, flight behaviour, circadian rhythms, adult responses to light, gustatory responses to sugar and sleep cycles (Cirelli et al., 2005; Ishimoto et al., 2005; Rodriguez Moncalvo & Campos, 2005; Ueda & Wu, 2006). The positional mapping of sex-linked mutations in D. melanogaster resulted in isolation of Sh101 (dominant) and Sh102 (recessive) shaker alleles that showed significant changes in adult flight behaviour compared to wild-type flies (Homyk, 1977; Homyk & Sheppard, 1977). D. melanogaster adult visual and gustatory responses were also significantly inhibited when shaker K\(^+\) channels were mutated or blocked to function improperly with tetanus toxin light chain (TNT; Ishimoto et al., 2005; Rodriguez Moncalvo & Campos, 2005). Finally, minisleep (mns) flies carrying a point mutation in the S1 domain of shaker had reduced lifespans and showed one-third of the wild type amount of sleep, but performed normally and were not functionally impaired by sleep deprivation (Cirelli et al., 2005; Yuan et al., 2006).

Peristaltic muscle contractions are rhythmic movements produced by the central pattern generators of the central nervous system in insects that are dependent upon feedback stimuli from the peripheral nervous system in order for coordinated contraction of body segments to occur (Caldwell et al., 2003). The D. melanogaster slmo gene is involved in coordination of peristaltic muscle contraction and movement behaviours in early instar larvae (Carhan et al., 2004). Mutant slmo phenotypes show a significantly reduced ability to recover from being flipped onto their dorsal surface, and have a reduced rate and number of peristaltic muscle contractions that result in an overall decrease in neonate movement (Carhan et al., 2004). Slmo is expressed within cells of the brain and ventral nerve cord of embryos and neonates, and the mitochondrial slmo protein has related proteins in Homo sapiens, Caenorhabditis elegans, Arabidopsis thaliana and Saccharomyces cerevisiae, suggesting that its neurological functions are highly conserved in eukaryotes (Nakai et al., 1994; Carhan et al., 2004; Fox et al., 2004; Dee & Moffat, 2005).

Understanding and identifying genes controlling pest behaviours and O. nubilalis larval movement in the field will aid in elucidating the effectiveness of current resistance management strategies and enhance our understanding of pest behavioural evolution in high-pressure transgenic Bt agricultural field environments. This study describes the mRNA features, predicted protein features, mRNA expression patterns and protein localization for Onshkr and Onsimo gene homologues in O. nubilalis. We have also evaluated and compared the genetic properties of Onshkr and Onsimo variants with reference to Drosophila and eukaryotic sequences and have aligned them phylogenetically with homologous sequences isolated from expressed sequence tag (EST) libraries of other lepidopteran species. These data help to provide a much needed foundation for studying the genetics controlling lepidopteran movement behaviours, and increase our understanding of how behavioural genetics may play an integral role in optimizing IRM strategies.

Results

Full-length cDNA sequences were assembled from 5’ and 3’ rapid amplification of cDNA ends (RACE) products for a single 1610 nucleotide (nt) transcript of Onshkr encoding a predicted protein product 469 amino acids (aa) in size (Fig. 1). The putative start codon, stop codon and polyadenylation for the encoded potassium channel transcript were indicated at nucleotide positions 21, 1428 and 1577 of the encoded transcript (Fig. 1). The Onshkr transcript possessed a short 21-nt 5’ untranslated region (UTR) and a 3’ UTR of 183 nt. Reverse transcription (RT)-PCR of the O. nubilalis life cycle and tissue samples indicated a
A. mRNA transcript and translation of *O. nubilalis* shaker

![Diagram of mRNA transcript and translation of *O. nubilalis* shaker gene](image-url)
constitutive high level of expression for Onshkr within all tissues and life cycle samples isolated from O. nubilalis (Fig. 1). Conserved domain database searches of the predicted O. nubilalis shaker protein identified conserved functional tetramerization and ion channel domains within the encoded protein sequence. The N terminal cytoplasmic tetramerization domain spans 89 amino acids (Fig. 1, green underlined sequence) and resembles the T1 domain of voltage-gated K+ channels (E-value 4.27E-33) and Broad-Complex, Tramtrack and Bric a brac (E-value 2.62E-07) superfamily proteins (Marchler-Bauer & Bryant, 2004; Marchler-Bauer et al., 2009). The C-terminal ion transport domain encompasses 262 aa (Fig. 1, blue underlined sequence) and encodes the six transmembrane helices found in Na+, K+ and Ca2+ ion channels (E-value 2.38E-10). Structural folding analyses indicated that the six-domain architecture was present in the ion channel domain (Fig. 2) necessary for the molecule to assume the three-dimensional (3D) structure of the known mammalian shaker K+ channel (Fig. 3). The O. nubilalis shaker protein consists of six putative transmembrane segments S1–S6 (Figs 2, 3). S4–S6 transmembrane segment sequences of O. nubilalis shaker are identical to the S6 sequences on D. melanogaster and rat shaker proteins (Baumann et al., 1988). The S4 sequences of O. nubilalis shaker, D. melanogaster shaker and rat shaker contain positively charged residues, arginine or lysine residues, that are located at every third position (Baumann et al., 1988).

Three splice variants (682, 970, 1604 nt) of Onsimo were identified in O. nubilalis neurological tissues encoding predicted proteins of 141, 228 and 228 aa, respectively (Fig. 4). The 5′ UTOR of all three transcripts varied with stop codons/polyA tails apparent at nucleotide positions 599/607, 860/941 and 860/1574, respectively, for variants 1, 2 and 3 (Fig. 4). The proteins encoded by variants 2 and 3 were identical, although Onsimo splice variant 3 possessed an extended 3′ UTR of 634 nt compared to variant 2 (Fig. 4). Although degenerate primer landing sites could not distinguish differential expression of the three splice variants, RT-PCR data demonstrated that Onsimo expression was constitutive in O. nubilalis from embryonic egg stages through adult emergence, and throughout all tissues of the fourth and fifth instars (Fig. 4).

Conserved domain database searches of predicted O. nubilalis slowmo proteins indicated that all three transcript variants encode the conserved 130–157 aa protein of relevant evolutionary and lymphoid interest/mitochondrial import-stimulation factor (PRELI/MSF1p’) functional domain (Fig. 5, black underlined sequence, E-value ≤ 5.46E-17) associated with the PRELI superfamily of mitochondrial proteins (Fig. 5). This superfamily of proteins encodes a conserved 170 aa domain predicted to function in intramitochondrial sorting (Anantharaman & Aravind, 2002). All O. nubilalis slowmo proteins aligned with the highly conserved domain extending for approximately 170 aa within the N-terminal region of the PRELI/MSF1p’ protein family (Anantharaman & Aravind, 2002) (Fig. 5).

Protein localization studies (Fig. 6) were performed in Trichoplusia ni High Five insect cells to observe protein properties of Onshkr and Onsimo translated products. As predicted, Onshkr proteins localized to the cell surface of T. ni High Five cells, suggesting a membrane-bound functional role for the protein in O. nubilalis (Fig. 6D–F). When expressed from High Five cells, the Onsimo protein localized to a perinuclear region (Fig. 6G–I) resembling the mitochondrial signals associated with...
other PRELI/MSF1p family proteins (Nakai et al., 1994; Carhan et al., 2004; Fox et al., 2004; Dee & Moffat, 2005).

To confirm further the relationships between *O. nubilalis* shkr and slmo proteins, and their homologous sequences, phylogenetic analyses were performed on Onshkr and Onslmo protein sequences. Phylogenetic alignment of the predicted Onshkr protein with homologous proteins and predicted homologous sequences from lepidopteran ESTs indicated that the lepidopteran sequences formed a single monophyletic group (Fig. 7A). As with Onshkr, the results consistently showed that Onslmo proteins also merged into one monophyletic group with other predicted lepidopteran slmo proteins (Fig. 7B).

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Drosophila melanogaster</em> shaker</td>
<td>P11651</td>
</tr>
<tr>
<td><em>Ostrinia nubilalis</em> shaker</td>
<td>ADV76532.1</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em> shaker</td>
<td>NP_001173.1</td>
</tr>
</tbody>
</table>

Figure 2. The predicted *Ostrinia nubilalis* shaker (Onshkr) protein shows structural homology to *Drosophila melanogaster* and *Rattus norvegicus* shaker proteins. Proposed transmembrane segments S1–S6 are highlighted in blue. National Center for Biotechnology Information protein accessions: *D. melanogaster* shaker, CAA29917.1; *O. nubilalis* shaker, ADV76532.1; *R. norvegicus* shaker, NP_001173.1.

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## Discussion

*Drosophila melanogaster* is a model system for the study of insect movement and behaviour, from which mutant strains showing a variety of abnormal locomotory traits have been identified. In this study, we have described the expression patterns, mRNA transcripts and encoded proteins for two neurological genes (*Onshkr* and *Onslmo*) isolated from *O. nubilalis* that show homology with known
between invertebrates and vertebrates and has been shown to be 82% identical between rat and *D. melanogaster* sequences (Baumann et al., 1988). The deduced protein structure of Onshkr also shows similarities with *D. melanogaster* and rat shaker proteins (Iverson et al., 1988), suggesting the putative function of *O. nubilalis* shaker as a voltage sensitive potassium ion channel (Figs 2, 3). The amino terminal sequence of the shaker protein probably plays a role in the physical gate whereas the carboxy terminus is important for channel inactivation (Timpe et al., 1988). In *D. melanogaster*, S1–S6 domains are transmembrane regions of the molecule, with S1–S4 forming the voltage-sensor module and S5–S6 the pore region (Iverson et al., 1988; Cirelli et al., 2005). The amino terminus of the transcript can vary but the central region S1–S4 is often highly conserved (Cirelli et al., 2005). It has been hypothesized that in an open voltage sensor conformation, the positive charged residues of S4 reach towards the extracellular solution (Long et al., 2007). In a closed conformation, S1 and S2 segments maintain position, the linker between S3 and S4 segments move inward, and positive charged S4 segment shifts towards the intracellular (Long et al., 2007). Our predictions of the structure and 3D model of *O. nubilalis* shaker indicated that it contains the six essential domains for proper folding and functioning of the K+ channel subunit (Figs 2, 3). Onshkr proteins also localized to the membrane surface when expressed from High Five insect cells, supporting a membrane-bound functional role for the proteins in *O. nubilalis* (Fig. 6). Further investigations are underway to determine physiological properties of Onshkr K+ channels in insect cells.

*Slmo* mutants adversely impact peristaltic motion and dorsal–ventral recovery in *D. melanogaster* (Caldwell et al., 2003; Carhan et al., 2004). Unlike the phenotypic effects observed in *shkr* mutants, central nervous system development and the excitation of motoneurons and muscles was normal in slmo mutants, suggesting that the slmo phenotype is not linked to neuronal development or nerve impulses (Carhan et al., 2004). *Slmo* is a member of a family of proteins containing a conserved PRELI/MSF1p’ domain that is known to be associated with several mitochondrial proteins including preli-like and real-time from *D. melanogaster* (Dee & Moffat, 2005). The mitochondrial colocalization of *D. melanogaster* slowmo, preli and real-time, mammalian preli, and *S. cerevisiae* MSF1p’ strongly suggests a biochemical function that is specific to the mitochondria (Nakai et al., 1994; Carhan et al., 2004; Fox et al., 2004; Dee & Moffat, 2005), although the specific functional role of the PRELI/MSF1p’ domain remains unknown. The three expressed *slmo* transcripts in *O. nubilalis* all carry protein coding sequences with the conserved PRELI motif (Fig. 5). Onslmo proteins localized to bright foci in...
A. mRNA transcript and translation of *O. nubilalis* slowmo

**Slowmo Ostrinia nubilalis variants 1, 2, 3**

1. AACCACCTGGT ATCACCACCA ATGCCCCAAG CAPTTCAATT CCAGCCGCTG CAPTTCAATT
   Onslow cDNA 1
2. AACCACCTGGT ATCACCACCA ATGCCCCAAG CAPTTCAATT CCAGCCGCTG CAPTTCAATT
   Onslow cDNA 2
3. AACCACCTGGT ATCACCACCA ATGCCCCAAG CAPTTCAATT CCAGCCGCTG CAPTTCAATT
   Onslow cDNA 3

51. GCCTTTCACT TGCAACCACG TCTACAGTTC ACCATTTAGT GAGGAAAAAG Onslow cDNA 1
51. GCCTTTCACT TGCAACCACG TCTACAGTTC ACCATTTAGT GAGGAAAAAG Onslow cDNA 2
51. GCCTTTCACT TGCAACCACG TCTACAGTTC ACCATTTAGT GAGGAAAAAG Onslow cDNA 3

101. AGGAAAATA TTTTTTAAAC AGGATTCCAG TCTACATCAG TAAATTACAG Onslow cDNA 1
101. AGGAAAATA TTTTTTAAAC AGGATTCCAG TCTACATCAG TAAATTACAG Onslow cDNA 2
101. AGGAAAATA TTTTTTAAAC AGGATTCCAG TCTACATCAG TAAATTACAG Onslow cDNA 3

151. AGGACCTGCG ATATATATAC ACAATTTAGT GAGGAAAAAG Onslow cDNA 1
151. AGGACCTGCG ATATATATAC ACAATTTAGT GAGGAAAAAG Onslow cDNA 2
151. AGGACCTGCG ATATATATAC ACAATTTAGT GAGGAAAAAG Onslow cDNA 3

FKHNPWLETVLVALALAWKL Onslow protein 1
FKHNPWLETVLVALALAWKL Onslow protein 2
FKHNPWLETVLVALALAWKL Onslow protein 3

251. CCCATCAATCC TGATATACC AGCACTTGGG ACCAGTGGT GAAAGAAGG Onslow cDNA 1
251. CCCATCAATCC TGATATACC AGCACTTGGG ACCAGTGGT GAAAGAAGG Onslow cDNA 2
251. CCCATCAATCC TGATATACC AGCACTTGGG ACCAGTGGT GAAAGAAGG Onslow cDNA 3

PNNPMNPAPTVGTQDVVER Onslow protein 1
PNNPMNPAPTVGTQDVVER Onslow protein 2
PNNPMNPAPTVGTQDVVER Onslow protein 3

301. AGTCTGACGC GCTACGCTG CTACACACCG ACTGCTAAGT TCTAAAGGG Onslow cDNA 1
301. AGTCTGACGC GCTACGCTG CTACACACCG ACTGCTAAGT TCTAAAGGG Onslow cDNA 2
301. AGTCTGACGC GCTACGCTG CTACACACCG ACTGCTAAGT TCTAAAGGG Onslow cDNA 3

VVDGVLHTHRLLSWSKWW Onslow protein 1
VVDGVLHTHRLLSWSKWW Onslow protein 2
VVDGVLHTHRLLSWSKWW Onslow protein 3

351. ATGATGACG AGGCCTAACG ATGCTAAGT TCTAAAGGG Onslow cDNA 1
351. ATGATGACG AGGCCTAACG ATGCTAAGT TCTAAAGGG Onslow cDNA 2
351. ATGATGACG AGGCCTAACG ATGCTAAGT TCTAAAGGG Onslow cDNA 3

FPRWAQALIGTAKICY FPRWAQALIGTAKICY FPRWAQALIGTAKICY
Onslow protein 1
Onslow protein 2
Onslow protein 3

401. GCAAGGAGAA AGGATCATGCT GATCCTAAGT CACATCCAAA TGAATGAGAA Onslow cDNA 1
401. GCAAGGAGAA AGGATCATGCT GATCCTAAGT CACATCCAAA TGAATGAGAA Onslow cDNA 2
401. GCAAGGAGAA AGGATCATGCT GATCCTAAGT CACATCCAAA TGAATGAGAA Onslow cDNA 3

ASEKSEVPIQRQMTLK ASEKSEVPIQRQMTLK ASEKSEVPIQRQMTLK
Onslow protein 1
Onslow protein 2
Onslow protein 3

451. GACAACACGC TTACATATGCT GCTGCTATG CTGCTGAGT GAGCAGTGA Onslow cDNA 1
451. GACAACACGC TTACATATGCT GCTGCTATG CTGCTGAGT GAGCAGTGA Onslow cDNA 2
451. GACAACACGC TTACATATGCT GCTGCTATG CTGCTGAGT GAGCAGTGA Onslow cDNA 3

TTNLFTFCRYEAVDETVK TTNLFTFCRYEAVDETVK TTNLFTFCRYEAVDETVK
Onslow protein 1
Onslow protein 2
Onslow protein 3
Figure 4. Transcript and mRNA expression analysis for *Ostrinia nubilalis slowmo*. (A) 5′ and 3′ rapid amplification of cDNA ends (RACE) of neurological cDNA yielded three transcripts of 682, 970 and 1603 nucleotides (nt) for the *O. nubilalis slowmo* mRNA encoding putative 141-, 228- and 228-amino-acid proteins with a conserved PRELI motif (underlined), respectively (GenBank: HQ116694, HQ116695, HQ116696). Locations of the putative start codons (green fill), stop codons (red fill), and polyA tails (grey fill) are indicated. The 1603-nt transcript encodes an extended 3′ untranslated region of 740 bp. Locations of landing sites for degenerate primers used in RT-PCR and the 5′/3′ RACE primers are indicated on the transcript maps. (B) Expression profiles for *slowmo* mRNA indicated constitutive high levels of expression in all life stages and tissues (see Fig. 1 for label descriptions) examined from *O. nubilalis*. 

B. *O. nubilalis slowmo* expression profile

<table>
<thead>
<tr>
<th>Life Cycle Screen</th>
<th>Tissue Screen</th>
<th>Neurological</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emb NN 2hd 3rd 4th 5th 6th Std</td>
<td>Std Cal</td>
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a perinuclear region consistent with that expected for a protein of mitochondrial origin (Fig. 6).

Phylogenetic alignment of Onshkr and Onslmo protein sequences with predicted and known proteins from homologous coding sequence (CDS) and EST sequences showed that both proteins aligned within monophyletic lepidopteran groups that were separate from other insect orders (Fig. 7A, B). The conservation of sequences associated with behavioural and neurological functioning within a single lepidopteran lineage is not surprising, given the similarities in life cycle events between all butterflies and moths. Conserved lineages and alignments also served to validate the genetic identities, transcript completeness and existence of homologous gene sequences retrieved from current lepidopteran EST databases (Fig. 7).

Conclusions

We are interested in uncovering and monitoring the genetic elements governing larval dispersal and movement behaviour in the European corn borer, as the ability of the larvae to disperse or move on transgenic plants is expected to directly impact the effectiveness of transgenic seed refuge strategies under evaluation for field release. The coordination of behavioural gene functions biologically is essential to proper ecological interactions in nature, and an alteration in any given one or multiple of these genes could be responsible for producing the behavioural phenotypes under selection in natural populations.

Investigations are underway to identify specific localization and functional attributes of Onshkr and Onslmo protein homologues. Additional studies are underway to determine if natural mutations in Onshkr and Onslmo exist, and how the expression patterns of these genes may change quantitatively between insects predisposed to differential behaviours and movement patterns in O. nubilalis populations. Single amino acid changes in the transmembrane domains of Shaker as well as subcellular ion concentrations can have strong impacts on protein functions and channel behaviour (Salkoff & Wyman, 1981; Armstrong & Miller, 1990; Perozo et al., 1992). In mns mutants of Drosophila, a single point mutation of C to T within the S1 domain alters a threonine residue to an isoleucine residue on the extracellular end of the S1 domain resulting in the short-sleep mutant phenotype (Cirelli et al., 2005). Site-directed mutagenesis of the seven basic amino acids and electrostatic analyses of the S4 domain of shaker indicated that this domain serves an

Figure 5. Multiple alignment of N-terminal region of Ostrinia nubilalis slowmo proteins and other members of the PRELI/MSF1p' protein family at widely diverged eukaryotic taxa. The O. nubilalis slowmo proteins contain conserved PRELI/MSF1p' domain that extends for approximately 170 amino acids on the Drosophila slowmo, Homo sapiens slowmo, preli, MSF1 and real time proteins (Anantharaman & Aravind, 2002; Dee & Moffat, 2005). GenBank protein accessions: O. nubilalis slowmo 1, 2, 3 (ADV76533.1, ADV76534.1, ADV76535.1); D. melanogaster slowmo (AAF23180.1); H. sapiens slowmo 1, 2 (AAI06751.1, AAH13969.1); D. melanogaster preli (NP_610487.1); H. sapiens preli (AAF09255.1); Saccharomyces cerevisiae MSF1-1 (EDV08125.1); Arabidopsis thaliana MSF1-1 (NP_196811.1); D. melanogaster real time (NP_600828.2).

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important role in mediating the voltage-dependent ion gating activity of the channel (Papazian et al., 1991; Lecar et al., 2003). The shkr locus is sex-linked to the Z chromosome in O. nubilalis and a single nucleotide polymorphism was discovered in natural population samples that can be used for genotyping of this locus in the field (Kroemer et al., 2011). Further analyses of the Onshkr and Onslmo loci may aid in revealing the overall complexity of these genes in O. nubilalis field populations.

Understanding the molecular basis for pest behavioural changes occurring in wild populations will aid in elucidating the effectiveness of current resistance management strategies and enhance our understanding of pest behavioural evolution in modern agricultural environments. Evaluating the genetic contributions to larval dispersal and movement in O. nubilalis will provide a foundation for elucidating the molecular interactions that govern pest behavioural adaptations and plant/insect interactions in agricultural systems.

**Experimental procedures**

*Nucleic acid isolation and cDNA synthesis*

Ostrinia nubilalis were obtained from a laboratory colony of bivoltine Z pheromone ecotype O. nubilalis (< six generations) maintained at the USDA-ARS, Corn Insects and Crop Genetics Research Unit (CICGRU), Ames, IA, USA. Life cycle samples: three replicates of O. nubilalis whole embryonic egg masses (four/replicate), pre-emergent black-head egg masses (four/replicate), second instars (30/replicate), third instars (10/replicate), fourth instars (two/replicate), fifth instars (one/replicate), pupae (one/replicate), male adults (one/replicate) and female adults (one/replicate) were collected from the USDA-ARS CICGRU laboratory colony. Samples were immersed in liquid nitrogen, ground into a powder, and then total RNA isolated in 500 ml Trizol reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). Tissue-specific samples: fourth and fifth instar O. nubilalis larvae were anaesthetized by immersion in ice-cold 1× phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ × 7H₂O, 1.4 mM KH₂PO₄, pH 7.4) for 20 min. Haemocytes were collected from 40 fourth and 40 fifth instar O. nubilalis larvae were anaesthetized by immersion in ice-cold 1× phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ × 7H₂O, 1.4 mM KH₂PO₄, pH 7.4) for 20 min. Haemocytes were collected from 40 fourth and 40 fifth instar larvae by cutting the first proleg with surgical scissors and bleeding into 500 ml ice-cold PBS, centrifuging at 800× g and washing three times in ice-cold PBS. Fat body from 40 individuals, digestive tracts (including gut and Malphigian tubules) from 10 individuals and remaining carcass tissues (including muscle, trachea, nerve cord and epidermis) from 10 individuals were dissected and placed in 500 μl ice-cold PBS. Purified tissues were centrifuged at 800× g for 5 min and washed three times with ice-cold PBS. Following washes, total RNA was isolated as described previously.

Oligo-dT primed 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 Virus (MMLV) reverse transcriptase (Promega Corporation, Madison, WI, USA).

**Figure 6.** Localization of Ostrinia nubilalis shaker (Onshkr) and slowmo (Onslmo) proteins. High Five cells were fixed and incubated in anti-V5 primary antibody as described in the Experimental procedures. (A) Phase contrast image for control (untransfected) cells. (B) Control cells stained with fluorescein isothiocyanate (FITC)-labelled secondary antibody. (C) Overlay image of control cells stained with FITC and 4′,6-Diamidino-2-phenylindole (DAPI). (D) Phase contrast image for cells expressing Onshkr. (E) Cells expressing Onshkr stained with FITC-labelleled secondary antibody. (F) Overlay image of DAPI and FITC signal from cells expressing Onshkr. (G) Phase contrast image for cells expressing Onslmo. (H) Cells expressing Onslmo stained with FITC-labelleled secondary antibody. (I) Overlay image for cells expressing Onslmo stained with FITC and DAPI. White arrows show individual cell expressing the Onshkr or Onslmo protein. Scale bars = 10 μm.

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Figure 7. (A) Phylogenetic analysis of *Ostrinia nubilalis* shaker (Onshkr) proteins. The representative phylogenetic tree of shaker proteins was constructed using the neighbor-joining method (Saitou & Nei, 1987). Shaker proteins that were identified from translated GenBank coding sequence (COS) or expressed sequence tag (EST) sequence matches are indicated. The evolutionary distances in the units of the number of amino acid substitutions per site are shown by the branch length with gaps eliminated. Phylogenetic analyses were conducted in MEGA 4.0 (Tamura et al., 2007). *Drosophila melanogaster* calcium-activated K+ channel protein (Blastx 10-5) was used as an outgroup for rooting the tree. GenBank protein accessions: *D. melanogaster* calcium-activated K+ channel (AAA28902.1); *Rattus norvegicus* shaker (NP_775118.1); *D. melanogaster* shaker isoforms A, B, C, D, E, F, G, H (AAN09452.1, AAF48785.3, AAF48790.2, AA09451.1, AAF48786.3, AAS65396.1, AAS65395.1, ACZ95322.1); *Tribolium castaneum* shaker (XP_001809693.1); *Pediculus humanus corporis* shaker (XP_002422887.1); *Aedes aegypti* shaker (XP_001660134.1); *Spodoptera frugiperda* shaker (FP364014.1); *Samia cynthia ricini* shaker (DC868623.1); *O. nubilalis* shaker (ADV76532.1). (B) Phylogenetic relationships of *O. nubilalis* slowmo (Onslmo) proteins. Slowmo proteins that were identified from translated GenBank coding sequence (CDS) or EST sequence matches are indicated. Branches with less than 50% bootstrap replicates are collapsed. Numbers indicate the percentage of replicate trees in which the associated proteins group together in the bootstrap test. The branch length represents units for the number of amino acid substitutions per site. *D. melanogaster* real-time, a divergent protein member of the PRELI/MSF1p' family, was used as outgroup for the phylogenetic analysis. GenBank protein accessions: *D. melanogaster* real-time (AAF52383.2); *Homo sapiens* slowmo (NP_057129.2); *N. vitripennis* slowmo (XP_001599085.1); *Culex quinquefasciatus* slowmo (XP_001849351.1); *A. aegypti* slowmo (XP_001660134.1); *Spodoptera frugiperda* shaker (FP364014.1); *Samia cynthia ricini* shaker (DC868623.1); *O. nubilalis* slowmo (ADV76532.1). (B) Phylogenetic relationships of *O. nubilalis* slowmo (Onslmo) proteins. Slowmo proteins that were identified from translated GenBank coding sequence (CDS) or EST sequence matches are indicated. Branches with less than 50% bootstrap replicates are collapsed. Numbers indicate the percentage of replicate trees in which the associated proteins group together in the bootstrap test. The branch length represents units for the number of amino acid substitutions per site. *D. melanogaster* real-time, a divergent protein member of the PRELI/MSF1p' family, was used as outgroup for the phylogenetic analysis. GenBank protein accessions: *D. melanogaster* real-time (AAF52383.2); *Homo sapiens* slowmo (NP_057129.2); *N. vitripennis* slowmo (XP_001599085.1); *Culex quinquefasciatus* slowmo (XP_001849351.1); *A. aegypti* slowmo (XP_001660134.1); *Spodoptera frugiperda* shaker (FP364014.1); *Samia cynthia ricini* shaker (DC868623.1); *O. nubilalis* slowmo (ADV76532.1).
multiple sequence alignments were performed using the CLUSTAL-accessions were imported into the MEGA 4.0 software suite (Tamura et al., 2007) separately for shkr and slmo genes, and multiple sequence alignments were performed using the CLUSTALW algorithm using default parameters [gap opening penalty 15, gap extension penalty 6.66, International Union of Biochemistry (IUB) weight matrix and transition weight of 0.5], and manually adjusted to correct for single base shifts. Degenerate oligonucleotides were designed manually to anneal at conserved nucleotide regions of the shkr and slmo genes, and the resulting primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA); ShkFW: 5′-CGG AAC GAR TAC TTC TTC GA-3′; ShkRV: 5′-CTT CAG CGA AGT ACA CCG C-3′; SlmoFW: 5′-GCA TGG CGA AAA TAT CCT AAT CC-3′; SlmoRV: 5′-AGC AAT GTA ATG RCA GAA WGT TAG GTT-3′.

Amplification and sequencing. 45-cycle PCR amplification reactions (96 °C–2 min initial denaturation, 96 °C–30 s cycle denaturation, 55 °C–45 s cycle annealing, 72 °C–2 min cycle elongation, 72 °C–10 min final elongation, 4 °C hold) were performed on cDNA pools and the products were visualized on 1.5% agarose gels containing 0.5

m/ml ethidium bromide. Amplified products were purified using a Gel/PCR DNA fragment extraction kit (IBI Scientific, Peosta, IA, USA), and ligated into the pGEM-T cloning vector (Promega Corp., Madison, WI, USA) at a 2:1 molar ratio as described by the manufacturer. The host Escherichia coli strain XL1-blue was transformed, selected on 50 mg/L ampicillin luria broth (LB) agar plates and clones containing inserts identified by blue/white screening. Plasmid DNA was purified using Epoch Plasmid Miniprep kits (Epoch Bioslabs, Missouri City, TX, USA) according to the manufacturer's instructions. Primer extension was performed using 1.6 pmol of the T7 or Sp6 primer with 0.1 μg plasmid DNA and products separated on a CEQ 8000 Genetic Analysis System (Beckman-Coulter, Brea, CA, USA) as described (Coates et al., 2008). The internal gene-specific primers (Shkr FW A: 5′-ATG CTG GAA CTG GGA CTG CTG-3′; Shkr FW B: 5′-ATC TTC AAG CTG TCT CGT CAC TCC-3′; Shkr RV A: 5′-CGC TTC GGT AGT AAT ACA GGA TGG-3′; Shkr RV B: 5′-CTC GGA GAA CAC GTG GAC GGG CAC-3′; Slmo FW A: 5′-TAT GCA AGT GAG AAA TCA GAA GTG-3′; Slmo FW B: 5′-CAA CTG CCA ATG ACA AGT AAAG ACA AC-3′) were designed for 5′ and 3′ RACE of cloned Onshkr and Onslmo transcripts. Analysis of a portion of the O. nubilalis genomic shaker locus was performed previously (Kroemer et al., 2011). Visual analysis of aligned genomic and cDNA sequences for Onshkr was used to identify the location of a single nucleotide polymorphism present within intron 2 in natural population samples of O. nubilalis.

Structural and function protein predictions

Functional protein domains were predicted by querying derived O. nubilalis peptides against the conserved domain database (Marchler-Bauer & Bryant, 2004; Marchler-Bauer et al., 2009). A 3D structural model of the O. nubilalis shaker protein was built using the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/), a protein structure prediction algorithm that combines the methods of threading, ab initio modelling and structural refinement to build models (Zhang, 2008, 2009; Roy et al., 2010). The protein structure for 2r9b (mammalian shaker potassium channel-beta subunit) was chosen as the template in the modelling. The confidence score (C-score), template modeling score (TM-score), and root median square deviation score (RMSD score) for the protein structure were calculated by I-TASSER. PyMOL software (Delano Scientific LLC, Palo Alto, CA, USA) was used for viewing the 3D structure of the modelled protein (DeLano, 2008).

Protein localization

Open reading frames of Onshkr and Onslmo were cloned into pBl/V5-His (Invitrogen, Carlsbad, CA, USA) by using KpnI (5′) and SacII (3′) restriction sites. T. ni (High-Five) insect cells (Invitrogen) were maintained in Express Five serum free medium (Invitrogen) supplemented with L-glutamine at 28 °C. Cells were plated at 75 to 80% confluence (1.6 x 10^6 to 2.5 x 10^6 cells/ml) in six-well plates. In vitro transfections of the recombinant pBl/V5-His vectors expressing the Onshkr and Onslmo gene constructs were performed according to the Cellfectin II (Invitrogen) transfection procedure with appropriate modifications where necessary. Briefly, 1 μg recombinant pBl/V5-His vector was combined with 1 ml serum free medium and 20 μl Cellfectin II reagent. The transfection mixture was incubated for 15 min at room temperature (RT) and overlaid onto the cells. Cells were incubated at RT for 4 h followed by the addition of 1 ml of the fresh medium and incubation at 28 °C for 72 h. Selection for stably transfected cells was performed with 50 μg/ml blasticidin–HCl (Fisher Scientific, Fair Lawn, NJ, USA) for 2 weeks. Blasticidin-resistant cells were cultured in flasks and continuously selected in Express Five medium containing 10 μg/ml blasticidin–HCl. Cells expressing Onshkr, Onslmo or untransfected cells were allowed to attach on glass microscope coverslips for 1 h at RT. 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 RT, and blocked for 1 h with PT plus 0.5% bovine serum albumin. The cells were incubated in a 1:100 dilution of

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analyses were conducted in MEGA 4.0 (Tamura and missing data were removed from the analysis. Phylogenetic tree was drawn from 1000 replicates (Felsenstein, 1985). All gaps joining method (Saitou & Nei, 1987) and the bootstrap consensus using tblastx algorithms. Evolutionary relationships amongst tide (nr) and nonhuman, nonmouse EST (est_others) databases transcripts were used as queries of NCBI random nucleotide polymorphisms (SNPs).

**Phylogenetic protein analyses**

*Ostrinia nubilalis* sequences were imported into MEGA 4.0 (Tamura et al., 2007) and multiple sequence alignments performed as described previously. Consensus *O. nubilalis* shkr and slmo transcripts were used as queries of NCBI random nucleotide (nr) and nonhuman, nonmouse EST (est_others) databases using tblastx algorithms. Evolutionary relationships amongst shaker and slowmo proteins were estimated using the neighbor-joining method (Saitou & Nei, 1987) and the bootstrap consensus tree was drawn from 1000 replicates (Felsenstein, 1985). All gaps and missing data were removed from the analysis. Phylogenetic analyses were conducted in MEGA 4.0 (Tamura et al., 2007). Predicted phylogenetic relationships were also constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sneath & Sokal, 1973), Minimum evolution (Rzhetsky & Nei, 1992), and Maximum parsimony (Eck & Dayhoff, 1966) methods to validate phylogenetic results.

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**References**


