**Supplementary material**

**AlepPBP2, but not AlepPBP3, may involve in the** **recognition of sex pheromones and maize volatiles in *Athetis lepigone***

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Running head: Functional binding properties of AlepPBPs

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**Supplementary Materials and Methods**

***Molecular cloning and preparation of expression vectors***

According to the previous method (Liu et al. 2015, Zhang et al. 2020), the genes encoding AlepPBP2 and AlepPBP3 were amplified, using primers with restriction sites for BamHI and XhoI at the 5′ and the 3′ ends, respectively. The PCR amplification was performed under the following conditions: 40 cycles at 98 °C for 10 s, 68 °C for 30 s. The reaction volume was as follows: 1 μL of stranded cDNA and 0.3 μM for each primer (upstream and downstream), 25 μL PrimeSTAR Max Premix (2×) (TaKaRa, Dalian, Liaoning, China) and 23.4 μL with nuclease-free water. After purification by agarose gel, the recycled products were cloned into the pEASY-T3 cloning vector (TransGen, Beijing, China), following the manufacturer's instructions. Then, three or four positive clones were selected for sequencing. After the results of sequencing were consistent with the target sequences, the returned plasmids and the expression vectors pET-30a(+) (Novagen, Darmstadt, Germany) were digested with BamHI and XhoI in the Fast Digest Green Buffer and again used the agarose gel to purify. Then, the purified products would be linked using the T4 ligase (Fermentas, Thermo Fisher Scientific, USA), according to the protocol. The vector plasmid sequences were determined to be correct by sequencing again, and the plasmid was used for expression of recombinant target proteins in BL21 (DE3) Escherichia coli cells.

***In vitro binding assays of AlepPBPs and data analysis***

The fluorescence competitive binding assays were performed on a Spectra Max M5 Fluorescence Spectrophotometer (Molecular Devices, USA) as our previous reported procedures (Zhang et al. 2020b). First, the different binding affinities of 1mM N-phenyl-1-naphthylamine (1-NPN) as the fluorescent reporter to two AlepPBPs were detected. The results were used to calculate the dissociation constants of 1-NPN to AlepPBPs, using the Scatchard plots of the binding data and assuming that two proteins were 100% active with a stoichiometric saturation obtained when the protein and ligand were equal. Then, 0.1mM sex pheromones or 1mM host plant volatiles were used to titrate the proteins that had been bound to 2 μM 1-NPN for exploring the competitive binding ability of ligands, referring to our previous experimental method for details (Zhang et al. 2014, Zhang et al. 2020). The relevant fluorescence intensities recorded was used to calculate the corresponding parameters, such as Ki and IC50, according to the previous analytical method reported and equation: Ki = [IC50] / (1 + [1-NPN]/K1-NPN). In the formula, IC50 values is the concentrations of the ligands halving the fluorescence of 1-NPN, [1-NPN] is the free concentration of 1-NPN and K1-NPN is the dissociation constant of the complex protein/1-NPN (Liu et al. 2013, Zhang et al. 2014). The data (mean ± SE) from various samples were subjected to a one-way nested analysis of variance (ANOVA) followed with the Tukey test for mean comparison (SPSS Inc., Chicago, IL, USA).

**References**

**Liu, N.Y., C.C. Liu, and S.L. Dong** (2013) Functional differentiation of pheromone-binding proteins in the common cutworm *Spodoptera litura*. *Comp Biochem Physiol A Mol Integr Physiol* 165, 254-262.

**Liu, N.Y., F. Yang, K. Yang, P. He, X.H. Niu, W. Xu, A. Anderson, and S.L. Dong** (2015) Two subclasses of odorant-binding proteins in *Spodoptera exigua* display structural conservation and functional divergence. *Insect Mol Biol* 24, 167-182.

**Zhang, X.Q., Q. Yan, L.L. Li, J.W. Xu, D. Mang, X.L. Wang, H.H. Hoh, J. Ye, Q. Ju, Y. Ma, M. Liang, Y.Y. Zhang, X.Y. Zhu, F. Zhang, S.L. Dong, Y.N. Zhang, and L.W. Zhang** (2020) Different binding properties of two general-odorant binding proteins in Athetis lepigone with sex pheromones, host plant volatiles and insecticides. *Pestic Biochem Physiol* 164, 173-182.

**Zhang, Y.N., Z.F. Ye, K. Yang, and S.L. Dong** (2014) Antenna-predominant and male-biased CSP19 of *Sesamia inferens* is able to bind the female sex pheromones and host plant volatiles. *Gene* 536, 279–286.

**Zhang, Y.N., J.W. Xu, X.C. Zhang, X.Q. Zhang, L.L. Li, X. Yuan, D.Z. Mang, X.Y. Zhu, F. Zhang, Y. Dewer, L. Xu, and X.M. Wu** (2020b) Organophosphorus insecticide interacts with the pheromone-binding proteins of *Athetis lepigone*: implication for olfactory dysfunction. *J Hazard Mater* 397, 122777.

**Supplementary Figure**

**Fig. S1.** **Binding curves of 1-NPN to AlepPBPs with relative Scatchard plots.**

